Epigenetics, MicroRNAs and the Activated Phenotype of Rheumatoid Arthritis Synovial Fibroblasts

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Table of contents

Zusammenfassung

Die meisten Menschen stellen sich unter „Rheuma“ altersbedingte Schmerzen in den Gelenken und durch Abnutzung verursachte Bewegungseinschränkung vor. Arthrose (oder Osteoarthritis) ist aber nur eine der vielen rheumatischen Erkrankungen, von denen allein in der Schweiz ca. 1,5 Millionen Menschen betroffen sind. Dabei ist „Rheuma“ keine Alterskrankheit. Dass alltägliche Bewegungsabläufe wie Kochen oder einen Brief schreiben zur schmerzhaften Herausforderung werden, kennen auch viele jüngere Menschen. Die rheumatoide Arthritis zum Beispiel, die häufigste entzündliche Gelenkserkrankung, tritt typischerweise ab dem dritten bis fünften Lebensjahrzehnt auf. Der Krankheitsverlauf ist meist chronisch und nur wenige Patienten können geheilt werden. Damit stellen die Krankheiten des rheumatischen Formenkreises nicht nur eine große individuelle Belastung dar, sondern verursachen auch enorme Kosten durch Arbeitsausfälle, medizinische Behandlungen, Begleiterkrankungen und z.T. auch eine verringerte Lebenserwartung.

Die rheumatoide Arthritis (RA) ist eine chronisch-entzündliche Systemerkrankung, die vor allem die Gelenke betrifft. Die fortschreitende Zerstörung von Gelenksknorpel und Knochen bereitet den Patienten große Schmerzen und eine zunehmende Bewegungseinschränkung. Eine wichtige Rolle bei der Krankheitsentstehung spielt das Immunsystem, welches sich gegen die körpereigenen Gelenkstrukturen richtet und gemeinsam mit lokalen Gelenkszellen Knorpel und Knochen angreift. Bei RA handelt es sich somit um eine Autoimmunerkrankung. Synoviale Fibroblasten (SF), also Bindegewebszellen in der Gelenkskapsel (Synovialis), sind in der RA die wichtigsten Zellen, die den Knorpelabbau vorantreiben; diese „RASF“ schütten knorpel-abbauende Enzyme aus, dringen tief in das Knorpelgewebe ein und unterhalten durch die Produktion von entzündlichen Signalmolekülen die chronische Gelenksentzündung. Da die RASF diese Eigenschaften auch zeigen, wenn sie aus der chronisch-entzündlichen Umgebung des RA-Gelenks herausgenommen werden (d.h. ohne weitere Stimulation ausserhalb des Körpers kultiviert werden), spricht man auch von einem aktivierten Phänotyp. Es gibt verschiedene molekulare Ursachen für diesen aktivierten Phänotyp, u.a. das Anschalten von Onkogenen (d.h. von Genen, welche die Entstehung und das Wachstum von Tumoren fördern) und das Ausschalten der entsprechenden Gegenspieler, der Tumorsuppressoren. Aus der Krebsforschung ist bekannt, dass für diese Veränderungen oft epigenetische Mechanismen verantwortlich sind. Das sind Vorgänge, die die Genexpression beeinflussen ohne dabei die Sequenz des genetischen Materials, der DNA, zu verändern, und die dennoch während der Zellteilung vererbt werden können. Genauer gesagt wird das Chromatin (die Speichereinheit der DNA in der Zelle bestehend aus DNA und Histonproteinen) durch verschiedene chemische Modifikationen lokal so verändert, dass Genexpression zugelassen oder unterbunden wird.

Eine weitere Instanz der Kontrolle der Genexpression, deren Bedeutung in den letzten Jahren immer deutlicher geworden ist, sind winzige RNA-Moleküle – microRNAs – von denen es über tausend verschiedene in menschlichen Zellen gibt. Eine bestimmte microRNA kann bis zu mehrere

hundert unterschiedliche Gene kontrollieren und somit das Verhalten von Zellen entscheidend beeinflussen.

Seit einigen Jahren gibt es immer mehr Hinweise dafür, dass der aktivierte Phänotyp der RASF durch epigenetische Veränderungen einerseits und durch die Fehlregulation verschiedener microRNAs andererseits bedingt wird. In meiner Doktorarbeit habe ich mich daher auf zwei bekannte Onkogene aus dem Feld der Epigenetik und aus dem Gebiet der miRNAs konzentriert, um deren Rolle in der Aktivierung der RASF zu untersuchen.

Im ersten Projekt habe ich die Histonmethyltransferase Enhancer of Zeste homologue 2 (EZH2) untersucht, die die Aktivität ihrer Zielgene durch Methylierung des Histon 3 an Lysinrest 27 (H3K27) unterdrückt. Ich konnte zeigen, dass RASF erhöhte Mengen von EZH2 exprimieren und dies durch Stimulation mit entzündlichen Signalstoffen (tumour necrosis factor α [TNFα]) noch verstärkt werden konnte. Ich konnte weiterhin herausfinden, dass EZH2 in RASF die Expression eines wichtigen Inhibitorproteins unterdrückt, das secreted frizzled-related protein 1 (sFRP-1). Der Genpromoter von *SFRP1* weist in RASF eine stärkere Methylierung von H3K27 auf und deshalb wird die Produktion von sFRP-1 unterbunden. Somit kann sFRP-1 den Wnt-Signalweg nicht mehr effektiv hemmen. Dieser ist in RASF aktiviert und trägt massgeblich zum aktivierten Phänotyp der RASF bei. Damit konnte ich mit diesem Projekt aufzeigen, dass die Histonmethyltransferase EZH2 durch epigenetische Regulation einen wichtigen Signalweg in der Pathogenese der RA beeinflusst.

Im zweiten Projekt beschäftigte ich mich mit einem microRNA-Cluster, der eine wesentliche Rolle im Immunsystem spielt und der, wenn er in bestimmten Immunzellen zu stark exprimiert wird, Autoimmunität hervorrufen kann. Dieser microRNA-Cluster – miR-17-92 – kodiert für sechs verschiedene microRNAs, die alle unterschiedliche Zielgene haben. In RASF wird die Expression von miR-17-92 durch Stimulation mit TNFα aktiviert, wobei dies vom NF-κB Signalweg abhängig war, einem der wichtigsten entzündlichen Signalwege, die bei RA dauerhaft aktiv sind. Um herauszufinden, welche Rolle einzelne microRNAs dieses Clusters in der Aktivierung der RASF spielen, wurden diese künstlich (d.h. durch Transfektion) in RASF eingebracht. Dabei konnte ich feststellen, dass miR-18a spezifisch die Expression von knorpel-abbauenden Enzymen und entzündlichen Mediatoren in RASF verstärkte. Mit Hilfe von Computer-basierten Vorhersageprogrammen wurden verschiedene mögliche Zielgene der miR-18a ermittelt. Durch die anschliessende experimentelle Validierung konnte der NF-κB-Signalweg-Inhibitor TNFα-induced protein 3 (TNFAIP3) als neues Zielgen der miR-18a identifiziert werden. Die Transfektion von miR-18a verstärkte somit das NF-κB-Signal in RASF. Zusammenfassend konnte so ein neuer positiver Feedback-Mechanismus entdeckt werden, bei dem TNFα den NF-κB-Signalweg wie auch die miR-18a induziert, wobei die miR-18a über die Hemmung des NF-κB-Inhibitors TNFAIP3 zu einer zusätzlichen Verstärkung des NF-κB-Signalwegs führt.

Summary

Most people associate "rheumatism" with age-related degenerative joint lesions leading to pain and limited mobility. Osteoarthritis, however, is only one of the many rheumatic diseases affecting 1.5 million people alone in Switzerland. Rheumatism is not restricted to diseases of the elderly. Even for younger people affected by these disorders activities of daily living such as cooking or writing a letter can become a painful challenge. The age at onset for rheumatoid arthritis (the most common inflammatory joint disease), for instance, is between the third and fifth decade of life. The course of the disease usually is chronic and only few patients can be cured. Thus, the rheumatic diseases do not only represent a great individual burden but also impose enormous costs upon society because of lost working hours, medical treatments, comorbidities and in some cases a reduced life expectancy.

Rheumatoid arthritis (RA) is a chronic-inflammatory systemic disease mainly affecting the articular joints. The progressive destruction of articular cartilage and bone causes pain and an increasing restriction of mobility. The immune system assumes an important role in the pathogenesis of RA; it turns against the body's own joint structures and, together with local synovial cells, attacks cartilage and bone. These processes define RA as an autoimmune disease. Synovial fibroblasts (SF), connective tissue cells of the joint capsule (synovium), are the major drivers of cartilage degradation in RA; these "RASF" secrete cartilage-degrading enzymes, invade deeply into the cartilage and maintain the chronic joint inflammation by the production of inflammatory signalling molecules. Since RASF retain these features even when they are taken out of the chronic-inflammatory environment of the RA joint (i.e. being cultivated outside the body without further stimulation) they are said to show an activated phenotype. This activated phenotype is based on several molecular aberrations, amongst others the activation of oncogenes (i.e. genes promoting the development and growth of tumours) and the switching-off of the respective opponents, the tumour suppressors. From cancer research it is known that these changes are often caused by epigenetic mechanisms. These are processes that influence gene expression without modifying the sequence of the genetic material, the DNA, but still may be inherited during cell division. More precisely, the structure of the chromatin (the storage unit of DNA in a cell consisting of DNA wrapped around histone proteins) is locally altered by different chemical modifications in such a way that gene expression is either permitted or restricted.

In recent years yet another instance of controlling gene expression has attracted growing attention: tiny RNA molecules – the microRNAs. A human cell can express more than thousand different microRNAs and one microRNA can control the expression of up to several hundreds of genes. Thus, microRNAs may significantly influence the behaviour of cells.

Epigenetic changes and the dysregulation of several microRNAs have been emerging as possible causes for the activated phenotype of RASF. During this doctoral thesis I have concentrated

on two well-known oncogenes from the epigenetics and the microRNA fields to investigate their role in the activation of RASF.

In the first project, I studied the histone methyltransferase Enhancer of Zeste homologue 2 (EZH2) which represses the activity of its target genes by methylating histone 3 at lysine 27 (H3K27). I could show that RASF express increased levels of EZH2 and that EZH2 is further inducible by stimulation with inflammatory mediators (i.e. tumour necrosis factor α [TNFα]). Furthermore, I could reveal that in RASF, EZH2 represses the expression of an important inhibitor protein, the secreted frizzled protein 1 (sFRP-1). The gene promoter of SFRP1 in RASF presents with stronger H3K27 methylation and hence the production of sFRP-1 is impaired. As a consequence, sFRP-1 cannot effectively inhibit the Wnt signalling pathway which is activated in RASF and decisively contributes to the activated phenotype of RASF. In conclusion, with this project I could show that through epigenetic regulation the histone methyltransferase EZH2 manipulates an important signalling pathway in the pathogenesis of RA.

Within the second project I addressed a microRNA cluster that plays a fundamental role in the immune system and, if overexpressed in certain immune cells, causes autoimmunity. This microRNA cluster – miR-17-92 – codes for six different microRNAs all of them having different target genes. In RASF, stimulation with TNFα activates the expression of miR-17-92. This was dependent on the NF-κB pathway, one of the most important inflammatory signalling pathways stably activated in RA. To elucidate the role of individual microRNAs from this cluster in the activation of RASF these were artificially introduced into RASF (i.e. by transfection). This allowed me to observe that miR-18a specifically enhanced the expression of cartilage-degrading enzymes and inflammatory mediators in RASF. With the help of computational prediction programmes various potential miR-18a target genes were sought. By subsequent experimental validation I could identify the NF-κB signalling inhibitor TNFα-induced protein 3 (TNFAIP3) as a novel target gene of miR-18a. Since TNFα is a major activator of NF-κB signalling in RASF this work discovered a new positive feedback mechanism: TNFα induces NF-κB activity and, thus, miR-18a which in turn represses the NF-κB inhibitor TNFAIP3 which further enhances NF-κB signalling in RASF.

CHAPTER I

Introduction

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease of the synovial joints affecting 0.5 to 1% of the population worldwide (1, 2). It is characterised by the persistent activation of the immune system, a continuous influx of immune cells into the joint and the activation of resident joint cells to degrade articular cartilage and bone. As a result, joints are progressively destroyed and become functionally impaired.

As with many other autoimmune diseases, RA is more common in women than in men. In a recent epidemiological study, the lifetime risk to develop RA was found to be 3.6% for women and 1.7% for men (3). The reason for the female preponderance is unresolved although sex hormones, microchimerism (i.e. the persistence of foetal cells in the mother) and X chromosome inactivation have been implicated (4, 5). There are significant regional differences in prevalence ranging from 0.2 to 5% between different continents and within countries pointing towards the involvement of environmental factors in the development of RA (6, 7). Despite decades of research in the field of RA the cause for this progressive and incurable disease has not yet been uncovered. It is generally accepted that a combination of genetic predisposition, environmental influences and possibly infectious agents lead to the development of RA. The best established genetic risk factor for RA is certain alleles of the human leukocyte antigen direct repeat B1 (*HLA-DRB1)* locus encoding the so-called shared epitope which is thought to break self-tolerance during antigen presentation (8, 9). Other well-established risk loci are the protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), peptidylarginine deiminase type 4 (*PADI4*), cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) and TNFα-induced protein 3 (*TNFAIP3*) genes. On a molecular level, the basis for the genetic association with RA is currently not clear for most of the genes; the majority of them are, however, connected to function or regulation of the immune system implying an actual functional role for these gene polymorphisms (9). Interestingly, there is also one *HLA-DRB1* allele (*HLA-DRB1*1301*) that confers protection from RA and is enriched in the healthy population (10). Despite the indisputable genetic association with RA the concordance rates for monozygotic twins are only ~15% and even less (~5%) for dizygotic twins (11). Therefore, other factors must play a role in the development of RA. Infection with certain viruses (e.g. Epstein-Barr virus [EBV] and retroviruses) or bacteria may have an influence on the pathogenesis of RA. The connection seems to be especially strong to periodontal disease as the bacterium *Porphyromonas gingivalis* expresses PADI4 and may therefore contribute to the generation of autoantigens (namely citrullinated proteins) (2, 12). Important contributors to RA are environmental influences, most importantly exposure to tobacco smoke. Especially smoking individuals carrying the shared epitope are considerably at risk to develop RA (13). Other factors may similarly decrease (e.g. consumption of alcohol, vitamin D intake and oral contraceptives) or increase (e.g. ingestion of red meat) the susceptibility to RA (13).

The socioeconomic and individual burden of RA is high. Because of the progressive joint destruction and systemic inflammation RA patients suffer from pain and disability as well as extra-articular comorbidities such as an increased risk to develop cardiovascular disease and depression. The consequences are a reduced quality of life in general, increased mortality, social isolation and high costs for the health care systems (8).

The diagnosis of RA is based on the American College of Rheumatology (ACR)/European League against Rheumatology (EULAR) classification criteria for rheumatoid arthritis of 2010 (14, 15). Patients presenting with joint swelling (i.e. active synovitis) and whose synovitis cannot be explained by any other diagnoses are eligible to be tested according to the criteria given in Table 1.

Table 1 The 2010 ACR/EULAR classification criteria for RA (adapted from Aletaha D *et al.* (14, 15))

Classification criteria for RA (score-based algorithm: add score of categories A-D) *a score of >6/10 is needed for classification of a patient as having definite RA*	Score
A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
≥10 joints (at least 1 small joint)	5
B. Serology (at least one test result is needed for classification)	
negative RF *and* negative ACPA	0
low-positive RF *or* low-positive ACPA	2
high-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least one test result is needed for classification)	
normal CRP *and* normal ESR	0
abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

RF = rheumatoid factor; ACPA = anti-citrullinated protein antibody; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate

These new criteria allow for an earlier diagnosis and, therefore, prompt and more rigorous intervention than the previous classification criteria of 1987 (16) which only diagnosed RA when patients already presented with the chronic erosive disease (14, 15). Thus, the current strategy is to initiate treatment as early as possible to prevent joint damage, suppress inflammation, control comorbidities and limit complications with the aim of achieving remission or even cure (17). One major concern with the new 2010 classification criteria is the problem of specificity meaning that patients might be misclassified as having RA when in fact having another disease (false-positive result) (18). As a consequence, biological samples from the early diagnosed RA patients (such as synovial tissue specimens) are more heterogeneous which might complicate their use in basic research studies. Human material used in this doctoral thesis has been obtained from RA patients diagnosed on the basis of the 1987 criteria. In

summary, the new classification criteria have to be considered in the light of early diagnosis and treatment intervention coming at the cost of reduced specificity (18).

After the diagnosis of RA, a disease-modifying antirheumatic drug (DMARD), usually methotrexate (MTX), is given as first-line treatment (8). Additionally, patients may receive a second DMARD or a biological agent in combination. In contrast to DMARDs and biologicals which limit joint swelling, systemic inflammation and joint destruction analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) merely treat the symptoms of RA, namely pain and stiffness (8). Glucocorticoids are indicated for short-term use only during flare-ups or locally (i.e. intra-articular) for particularly active joints because of the substantial unwanted effects they cause (8). The use of biologicals has revolutionised RA treatment in the last decades; blocking cytokines/cytokine signalling and immune cell activation has been shown to be highly effective in limiting the progression of RA. Currently in clinical use are biologicals that block tumour necrosis factor (TNF)-α (Adalimumab, Certolizumab pegol, Etanercept, Golimumab and Infliximab), interleukin (IL)-1β (Anakinra) and IL-6 (Tocilizumab), inhibit T cell activation (Abatacept) and deplete B cells (Rituximab). Other strategies targeting for example IL-17, IL-23/IL-12-p40 or IL-15 are under investigation (17). As alternative to MTX and other DMARDs such as hydroxychloroquine, sulfasalazine and leflunomide, new small molecule inhibitors of intracellular kinases such as Janus kinase (JAK) or spleen tyrosine kinase (SYK), are in development to be used as novel DMARDs (8).

Despite all the progress that has been made in the treatment of RA principal obstacles are yet to be overcome. Although remission is achieved more frequently RA remains an incurable disease. Therefore, patients need to have regular examinations and take life-long medication (8). Often, disease flare-ups cannot be prevented and in many cases, drugs have significant unwanted effects, for instance higher susceptibility to opportunistic infections, in particular to mycobacterial disease under TNFα blockade. Since biologicals are extremely expensive their cost-effectiveness is a matter of debate and the search for more affordable alternatives is on-going. Finally, there are still a considerable number of patients who do not or insufficiently respond to therapy (2, 8). For these reasons, research to find the cause of RA, to better understand its pathogenesis and to develop novel therapies targeting the disease must continue.

Pathogenesis –many components in a multifactorial disease

Rheumatoid arthritis is a very complex disease with various cell types and biological processes involved. Beside immune cells immigrating from the blood stream resident joint cells (i.e. fibroblasts, macrophages, chondrocytes, endothelial cells and osteoclasts) act in perpetuating joint inflammation and executing cartilage and bone destruction.

The capsule of healthy diarthrodial joints is lined by a thin synovial membrane (the synovium) consisting of the 1-2 cells strong lining layer (composed of macrophages - type A - and fibroblasts -

type B synoviocytes) and a rather a-cellular sublining mainly made up of loose connective tissue. The function of the synovium is lubrication of the joint cavity and supplying chondrocytes with nutrition and oxygen (19, 20). In RA, the lining layer thickens (up to 15 cell layers), the sublining is populated by immune cells attracted from the circulation and angiogenesis (i.e. the formation of new blood vessels) takes place. Altogether, these mechanisms lead to the formation of the destructive pannus tissue (1, 2, 19) (Figure 1). Resident and immigrated cells in the synovium cooperate in perpetuating joint inflammation and damage as summarised in Figure 2 (2).

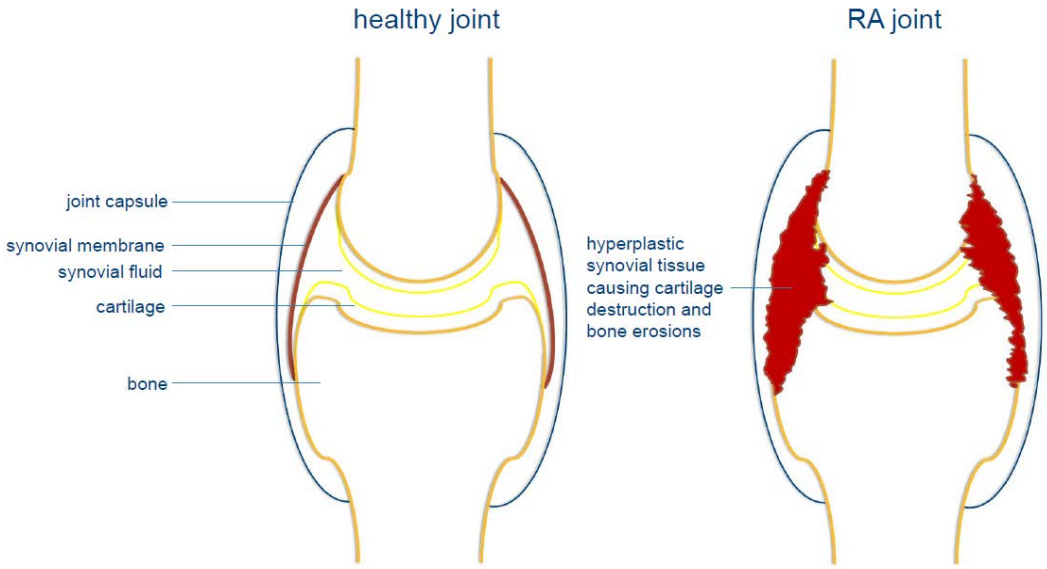

Figure 1 Schematic representation of a healthy and a RA joint

In RA, proliferation and reduced apoptosis of cells within the synovial membrane as well as continuous extravasation of circulating immune cells into the joint tissue lead to the formation of pannus tissue that destroys cartilage and bone.

The autoimmune reaction inside the rheumatoid joint comprises the innate and the adaptive compartment. The innate immune response, apart from anatomical barriers like skin and mucous membranes, is the first line of defence against pathogens and provides the basis for the antigen-specific reactions of the adaptive immune system (as procured by the action of B and T cells). Pathogen-associated molecular patterns (PAMPs) derived from the intruder (e.g. DNA, RNA, peptidoglycan) activate pattern-recognition receptors (PRRs) on the surface of innate immune effector cells and initiate a cascade of events including the secretion of cytokines and chemokines, activation of the complement system, extravasation to the site of infection, pathogen clearance by phagocytosis, killing of virus-infected cells, and lastly, antigen presentation to activate adaptive mechanisms of defence (21). The major innate immune cells inside the RA joint are macrophages, but natural killer cells, mast cells, neutrophil granulocytes, and dendritic cells are abundant as well (2). Mast cells, best known for the release of histamine during allergic reactions, are implicated in cytokine production and neutrophil recruitment to the RA synovium (22). Neutrophils, which are mainly found in the synovial fluid of RA patients, are defective in apoptosis and release high amounts of reactive oxygen and nitro-

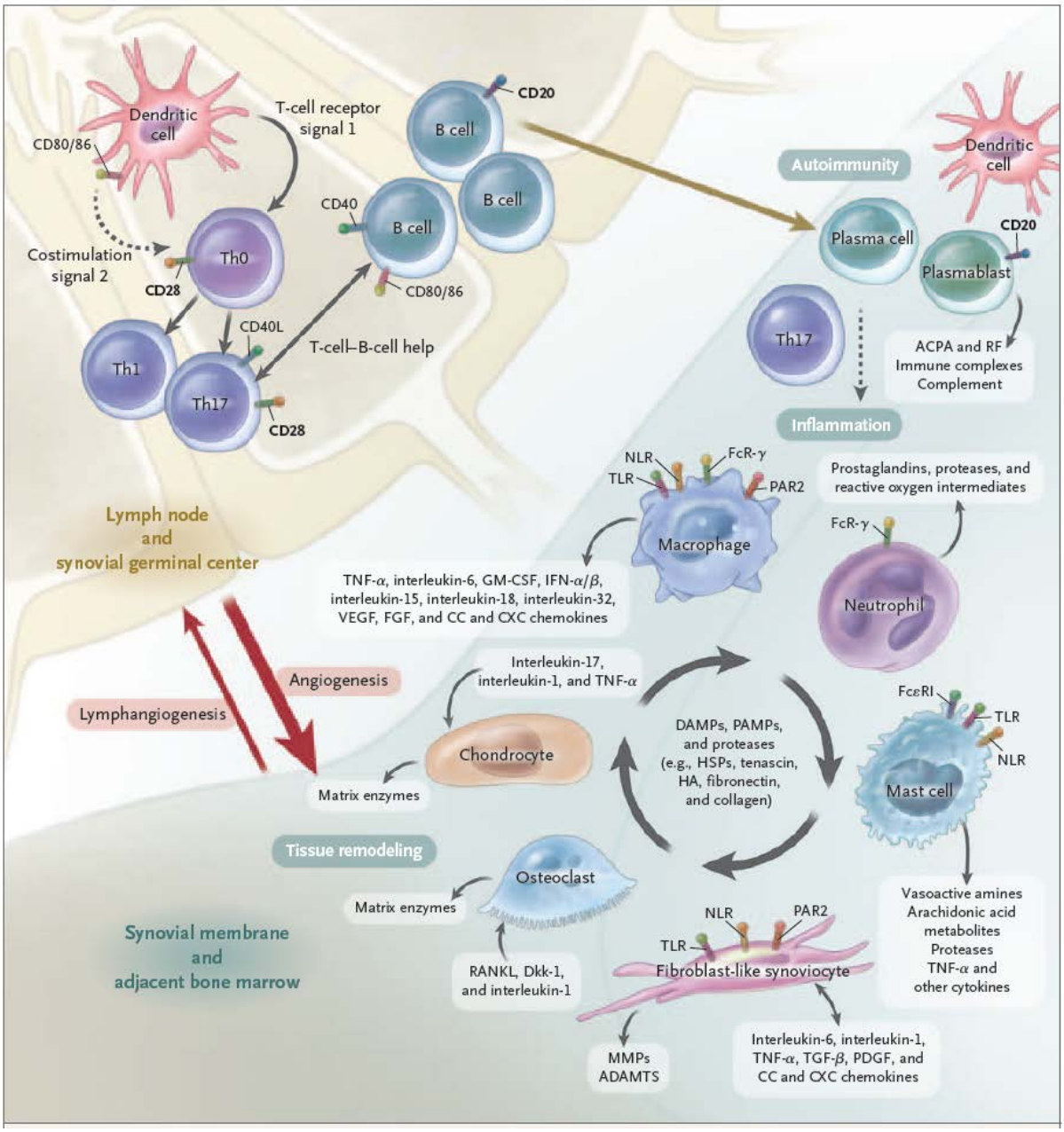

Figure 2 Pathogenic processes in the RA joint mediated by different cell types (from McInnes IB and Schett G (2))

Inside the RA synovium, resident cells as well as cells of the innate and the adaptive immune system promote chronic inflammation and joint destruction. Locally produced inflammatory cytokines and chemokines activate and attract leukocytes from the blood stream. Synovial fibroblasts and osteoclasts actively destroy cartilage and bone. The increased metabolic demand causes hypoxia which initiates angiogenesis allowing the influx of an even greater amount of immune cells. Hypoxia, degradative and apoptotic processes generate DAMPs to further activate cells in the synovium altogether generating a vicious cycle of mutual activation of resident and immigrated cells of the joint.

Abbreviations: ADAMTS a disintegrin and metalloprotease with thrombospondin-1-like domains, DAMP damage-associated molecular pattern, Dkk-1 Dickkopf-1, FcR Fc receptor, FcεRI high affinity IgE receptor, FGF fibroblast growth factor, GM-CSF granulocyte-macrophage colony-stimulating factor, HA hyaluronan, HSP heat-shock protein, IFN-α/β interferon-α/β, MMP matrix metalloproteinase, NLR nucleotide-binding oligomerization domain-like receptor, PAMP pathogen-associated molecular pattern, PAR2 protease-activated receptor 2, PDGF platelet-derived growth factor, RANKL receptor activator of nuclear factor κB ligand, TGF-β transforming growth factor β, Th0 type 0 helper T cell, Th1 type 1 helper T cell, Th17 type 17 helper T cell, TLR toll-like receptor, TNF-α tumour necrosis factor α, VEGF vascular endothelial growth factor

gen species (ROS and RNS) as well as proteases and inflammatory mediators thereby augmenting synovial inflammation and tissue injury (2, 23). Dendritic cells (DC) are the major professional antigen-presenting cells (APCs) which, following their activation, start to differentiate and migrate to the lymphoid tissues where they display antigens *via* the major histocompatibility complex (MHC) molecules to effector cells (24). The role of DC in RA pathogenesis is ambiguous; depending on the subset they may be involved in maintaining (plasmacytoid DC) or breaking (classical DC) self-tolerance, and numbers of circulating DC (both plasmacytoid and classical) were differentially modulated by various therapeutic interventions in RA patients (24).

Macrophages (Mϕ) are tissue-specific monocyte-derived phagocytes specialised in the removal of pathogens and cellular debris. They are key producers of the most crucial RA-associated cytokines – TNFα, IL-1β and IL-6. Targeting Mϕ and their products has proven especially successful in treating RA; Mϕ numbers in the sublining were shown to be reduced by DMARDs such as MTX or leflunomide and TNFα antagonists (24). Similarly, blocking IL-1β or IL-6 significantly improves the disease course. These facts underpin the importance of Mϕ in the pathogenesis of RA. Elevated numbers of Mϕ in the RA synovium contribute to joint destruction at several levels: beside a plethora of cytokines they are also a source of chemokines attracting more immune cells from the blood stream, they release ROS, RNS and matrix-degrading enzymes, phagocytose and present antigen, and drive bone erosions after differentiating into osteoclasts (2, 25, 26). Healthy bone is constantly remodelled by the simultaneous action of bone-forming osteoblasts and bone-resorbing osteoclasts. In RA, this finely-tuned balance is skewed towards an overrepresentation of osteoclasts and therefore undue bone resorption (27). In the presence of certain stimuli, in particular macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), hematopoietic stem cells differentiate towards the monocytic/macrophage lineage to form osteoclast progenitor cells which finally fuse to become fully differentiated multinucleated osteoclasts (28). In the RA synovium, RANKL is abundantly expressed. Together with the high amounts of pro-inflammatory cytokines such as IL-6 and TNFα this is thought to cause the imbalance in bone remodelling and articular bone loss in RA (27, 28).

Innate immune cells are activated through the binding of PAMPs to PRRs – toll-like receptors (TLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Different TLRs and NLRs are expressed throughout the synovium, not only in Mϕ but also in synovial fibroblasts, and particularly the expression of TLR2, 3, 4, 7, and 8 as well as NOD2 has been associated with proinflammatory and destructive activity (24, 29). The evidence for the presence of actual disease-causing PAMPs in synovial tissue is scarce; despite the fact that infections may be connected to RA pathogenesis and viral or bacterial DNA may be present in synovial tissue these could not clearly be pinned down as being true initiators of RA (29). Instead, endogenous ligands for TLRs – damage- or danger-associated molecular patterns (DAMPs) – have been found in great variety and abundance. These molecules are unleashed upon tissue injury and thus link sterile tissue damage and inflammation

(30). DAMPs include but are not limited to heat shock proteins (HSP), lipoproteins such as serum amyloid A, alarmins as high-mobility group box-1 protein (HMGB1), self-nucleic acids released from necrotic cells, and extracellular matrix (ECM) components (e.g. hyaluronic acid fragments) (30). With the enduring tissue destruction in RA there is a sustained supply with DAMPs and thus, the interest in developing means of antagonising TLR signalling in RA has resulted in several preclinical and clinical trials (29).

During an infection, the innate immune response is followed by the antigen-specific adaptive response mediated by B and T lymphocytes. Aberrant activation of T lymphocytes is a characteristic of RA pathogenesis which is also exemplified by the success of therapies inhibiting T cell activation (Abatacept) (17). Once the T cell receptor (TCR) interacts with MHC-bound antigens on APCs T cells become activated. Additionally, the APC needs to provide costimulatory signals of the B7 family of immunoglobulins (cluster of differentiation [CD]80 and CD86) which bind to CD28 and CTLA4 on the surface of the T cell (21). Abatacept containing the extracellular part of CTLA4 binds to CD80/CD86 and thereby prevents costimulation *via* CD28 and thus inhibits T cell activation (17). RA has classically been viewed as a Th1 disease (i.e. a predominance of cytokines derived from T helper cells type 1 [Th1] and paucity of Th2 cytokines) but this view has been challenged by data showing beneficial effects of interferon-γ (IFN-γ) and other Th1 cytokines in RA animal models and *in vitro* (31). With the discovery of new T cell subsets, in particular the Th17 and regulatory (Treg) T cells, the T cell compartment in RA has regained attention in recent years. Whereas Treg ($CD4^+CD25^+$ cells that express the transcription factor forkhead box protein P3 [Foxp3]) have immunosuppressive activity which appears to be attenuated in RA (32) Th17 ($CD4^+CD25^-$ cells that uniquely express the transcription factors retinoic acid receptor-related orphan receptor-γ-t [RORγt] and RORαt) have strong proinflammatory functions. Th17 are a major source of the IL-17 family of cytokines which exert pleiotropic functions in the activation of immune cells and synoviocytes, osteoclastogenesis and angiogenesis. In this manner, IL-17 directly contributes to the major hallmarks of RA – inflammation, joint infiltration, cartilage destruction and bone erosions – and therapeutics targeting IL-17 are underway (31).

B cells are attracted to the RA joint by locally produced chemokines, and in the synovium they are often organised in ectopic germinal centres together with T lymphocytes and dendritic cells (33, 34). They produce a range of inflammatory cytokines and chemokines and function as APCs for T cells thereby contributing to the maintenance of the chronic inflammatory state of the synovium. Their main function in RA, however, is the production of autoantibodies (34). Beside the long-known association of rheumatoid factor (RF; i.e. autoantibodies directed against an epitope on the constant fraction of immunoglobulin G) with the disease, the new classification criteria for RA also include other autoantibodies, the anti-citrullinated protein antibodies (ACPA) (14, 15, 33). ACPA-positive RA is associated with other genetic risk loci and environmental risk factors than ACPA-negative RA (9, 13). Patients positive for both ACPA and RF bear a worse prognosis and, thus, ACPA-positive RA

may represent a clinical entity distinct from the autoantibody-negative disease (8, 9, 33). B cells in RA are currently targeted by Rituximab, a B cell depleting chimeric monoclonal antibody for CD20 (17, 33). Interestingly, CD20 can only be found on pre-B to mature B cells but not on the antibody-producing plasma cells and the mode of action of Rituximab may mainly be imputable to the control of cytokine production, T cell activation and reduced development of memory B cells after repopulation (17, 33).

A further pathogenetic feature of RA is dysregulated synovial angiogenesis. Because of the constant influx of immune cells alongside increased proliferation and decreased apoptosis of synovial cells the metabolic demand inside the synovium outpaces the existing vascular supply (35, 36). Still, despite the strong angiogenic drive in the synovial tissue the newly formed vessels appear to have maturation defects, i.e. the recruitment of pericytes (mural cells that stabilise the endothelial tubes) is compromised which prevents optimal perfusion (37). Therefore, the synovial microenvironment remains hypoxic and leaky vessels facilitate constant immune cell infiltration. Together, these events result in a vicious cycle of sprouting angiogenesis, hypoxia and immune cell extravasation (35).

Rheumatoid arthritis synovial fibroblasts – the tumour-cell like invasive phenotype

The work in this doctoral thesis has concentrated on the rheumatoid arthritis synovial fibroblast (RASF), and, thus, this section shall be dedicated to this cell type. Considered as an innocent bystander for many years RASF are now recognised as the driving force in cartilage destruction in RA. The prominent role of RASF in the degradation of articular cartilage became apparent for the first time when fibroblasts from different sources (i.e. RA, osteoarthritis [OA] or normal synovium or skin) were co-implanted with human cartilage into severe combined immunodeficient (SCID) mice and the cartilage was analysed after 60 days (38). In contrast to OA, normal SF or skin fibroblasts RASF were able to deeply invade into the cartilage, and this went along with the expression of matrix-degrading enzymes and adhesion molecules, particularly at the site of invasion (38). This study proved for the first time that RASF are capable of driving tissue destruction independent of a fully functional immune system. Ever since, RASF have been the subject of many studies trying to elucidate the reasons for their aberrant activation and “transformation”. RASF share many features with malignant cells, i.e. defects in controlling proliferation/apoptosis and (local) invasiveness (1, 39, 40). This “activated phenotype” of RASF may be a consequence of the increased expression of anti-apoptotic molecules (e.g. Bcl family members or the small ubiquitin-like modifier [SUMO]-1) and proto-oncogenes (e.g. c-Myc) and inactivation of tumour suppressor genes (e.g. p53 or phosphatase and tensin homologue [PTEN]) (1, 39). Furthermore, they express a range of adhesion molecules mediating their binding to cartilage matrix or cell-cell interactions; these include members of the integrin family, vascular-adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and cadherin-11 (19, 41). The similarities of RASF with cancer cells and the invasive behaviour of the pannus tissue

might allow for RA to be viewed as a locally invasive tumour with RASF being described as tumour-cell like or transformed (1, 20, 42). Interestingly, the importance of the migratory/invasive RASF in the pathogenesis of RA has recently been further substantiated by data demonstrating that RASF injected subcutaneously into SCID mice migrate towards and invade into co-implanted human cartilage at distant sites (43) which was interpreted as a form of metastasis by the authors (44). Despite the fact that nowadays we know a lot about the imprinted phenotype of RASF the molecular mechanisms responsible for the transition from the healthy to the RASF are largely unknown (45).

Additionally to their apparent intrinsic activation, RASF can be stimulated by a great range of inflammatory mediators (i.e. cytokines and chemokines), TLR ligands, cartilage and ECM components, as well as the direct physical interaction with immune and other cells, all of which can be found in the RA synovium (19). The result of RASF activation is the production and secretion of matrix metalloproteinases (MMPs) and other cartilage-destructive molecules, and pro-inflammatory cytokines and chemokines acting in autocrine and paracrine ways to fuel joint inflammation and destruction (19). Some especially important activators and products of RASF are shown in Table 2.

Table 2 Stimuli activating RASF and pro-inflammatory and matrix-degrading molecules produced by RASF (adapted from Noss EH & Brenner MB (19))

RASF activators	RASF products
cytokines (TNFα, IL-1β, IL-6, IL-17, IFN-γ, IL-18)	cytokines (IL-6, IL-15, IL-23, type I interferons)
growth factors (FGF, PDGF, TGF-β)	growth factors (GM-CSF, VEGF, TGF-β, PDGF)
chemokines (CCL-2, CCL-5, CCL-13, CXCL-12, CX3CL-1)	chemokines (CXCL-1, -5, -6, -8, -9, -10, -11, -12, -12, CCL-2, -3, -5, CX3CL-1)
bioactive lipids (prostaglandins, leukotrienes)	bioactive lipids (prostaglandins)
cell surface ligand interactions (CD40/CD40L, VLA-4/VCAM-1, LFA-1/ICAM-1)	cell surface receptors (CD40, VCAM-1, ICAM-1, TLRs, integrins, cytokine and chemokine receptors)
tissue degradation products (DAMPs, fibronectin and thrombin fragments, microparticles)	degradative enzymes (MMPs, cathepsins)
hypoxia	

FGF = fibroblast growth factor, GM-CSF = granulocyte-macrophage colony-stimulating factor, LFA-1 = lymphocyte function-associated antigen-1, PDGF = platelet-derived growth factor, TGF-β = transforming growth factor β, VLA-4 = very late antigen-4

As resident cells of the synovium, RASF get into bidirectional contact with all other synovial cells (local and immigrated), either directly *via* cell surface interactions or indirectly *via* secreted products (reviewed in 19) (Figure 3). RASF are activated by inflammatory cytokines and, in response, secrete cytokines themselves; these are able to activate immune cells in the synovium to produce even more cytokines. At the same time, RASF release chemokines that attract more leukocytes from the blood stream. Direct contact of RASF with immune cells further promotes synovial cell activation (19, 20). Certain soluble mediators produced by RASF such as VEGF or PDGF stimulate synovial angiogenesis (46) which, as mentioned earlier, seems to be defective in general and, moreover, facili-

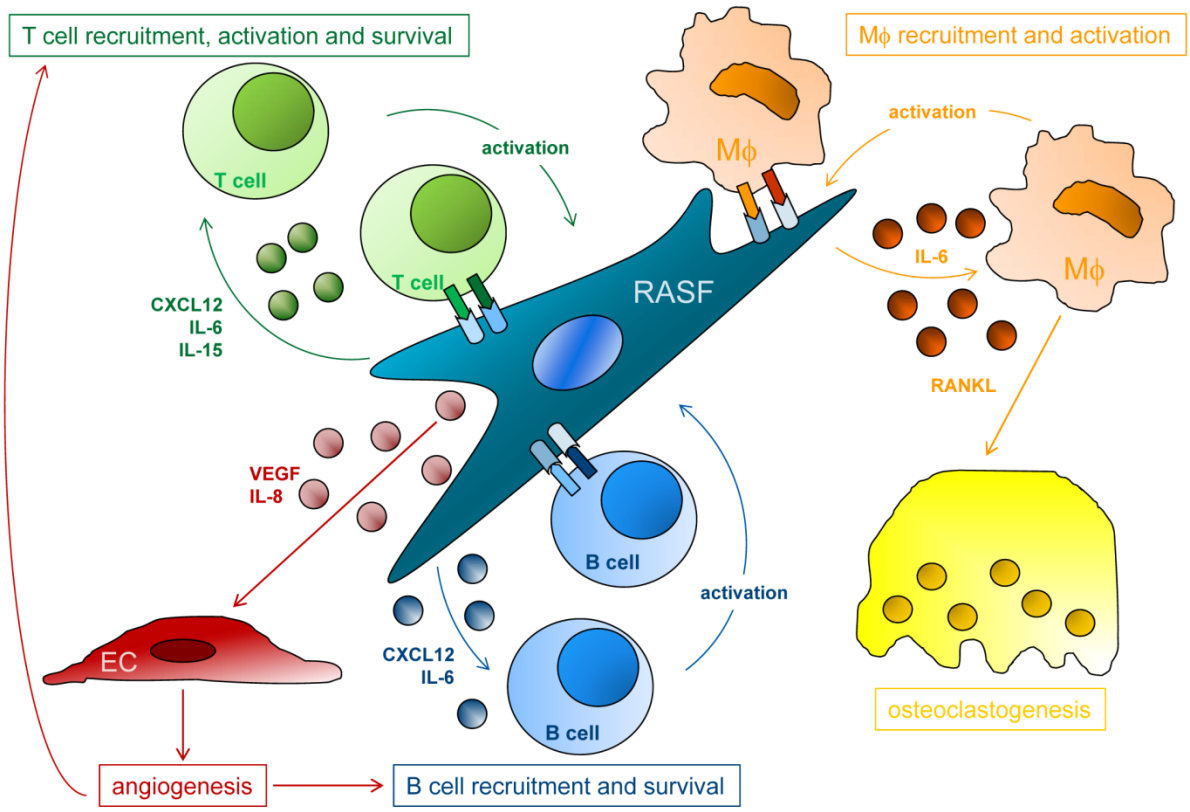

Figure 3 Cellular interactions of RASF

Through the secretion of cytokines and chemokines as well as direct cell-cell contacts (mediated by integrins, adhesion molecules and cell surface molecules acting as co-stimulatory factors) RASF recruit and activate circulating leukocytes, resident macrophages and promote osteoclast differentiation. Leukocyte extravasation is facilitated by increased angiogenesis which is also supported by RASF. In turn, RASF are activated by leukocyte- and Mϕ-derived products. Abbreviations: EC endothelial cell, Mϕ macrophage, CXCL12 chemokine (C-X-C motif) ligand 12, IL interleukin, VEGF vascular endothelial growth factor

tates immune cell infiltration because of vessel leakage (37). Activated RASF are a yielding source of RANKL and therefore are important promoters of osteoclastogenesis and bone erosions (27, 47). Thus, the RASF appears to be at the heart of the events creating an environment of chronic joint inflammation, cartilage destruction and bone erosions.

Cytokine and signalling pathway networks in rheumatoid arthritis

Cytokine signalling networks are of utmost importance in the development of RA being crucial regulators of autoimmunity, chronic synovitis and synovial tissue destruction (48). Articular cells express and secrete a great variety of cytokines that activate the secreting cell itself (autocrine) and/or other cell types (paracrine) triggering proinflammatory and destructive interactions that ultimately perpetuate joint inflammation and destruction (48). TNFα, IL-1β and IL-6 are considered the major cytokines involved in RA pathogenesis. Mainly produced by activated macrophages, TNFα influences all pathological processes in the RA joint; it causes or supports the production and release of proinflammatory cytokines, chemokines, matrix-degrading enzymes and prostaglandins, endothelial

cell activation, angiogenesis, leukocyte accumulation and osteoclast activation (49, 50). IL-6 acts on leukocyte differentiation and activation, autoantibody production and is the prime inducer of the acute-phase response; it has been associated with RA-related osteoporosis as well as other systemic complications such as cardiovascular disease, fatigue and depression (51). IL-1β promotes synovial inflammation and - being an extremely effective inducer of matrix-degrading enzymes and a direct activator of bone-resorbing osteoclasts - is a major driver of tissue destruction; it is estimated that in relation to TNFα IL-1β is more important in the destructive processes whereas TNFα is the major driver of inflammation (52). Other cytokines that have more recently attracted the attention of the RA community are IL-15, IL-17, IL-21, IL-23, and IL-33. Especially, the field of research on the role of IL-17 in RA has exploded during the last years and IL-17 was shown to contribute to SF activation, osteoclast differentiation, immune cell infiltration, and synovial angiogenesis (53). In different RA animal models, IL-17 blockade generated varying responses and three different antibodies targeting IL-17 signalling are currently in clinical trials (17, 53). Importantly, the expression of cytokines is interdependent; TNFα, for instance, stimulates the expression of IL-6 in SF which in turn activates Mϕ (the main source of TNFα and IL-1β) and contributes to the development of IL-17-producing Th17 cells. Thus, cytokines and the cells they are produced by and act on form a dense network of interactions, synergisms and antagonisms that ultimately result in the proinflammatory joint environment of RA.

Of interest, in many cases it seems that blocking one single cytokine (as in the case of TNFα) is sufficient to achieve amelioration of inflammation and thereby clinical improvement in RA patients, a concept which had been widely rejected when it was initially proposed by Feldmann and Maini in the early 1990s (54). Thus, one can assume that cytokine networks become disrupted if one component is missing making it easier to target this vicious cycle of proinflammatory actions. Still, a certain hierarchy appears to exist as blocking TNFα and IL-6 signalling shows the greatest clinical benefit whereas IL-1 blockade has comparably modest effects (17, 55) proving some cytokines "more equal than others"[1].

One cannot look at cytokines without examining the downstream signalling they initiate. Once bound to their receptor(s), cytokines activate many different signalling pathways of which the nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), JAK – signal transducer and activator of transcription (STAT) and phosphatidylinositol 3-kinase (PI3K) pathways are considered to be among the most important in RA (2). These pathways subsequently activate cell-type specific gene transcription programs that eventually contribute to disease pathogenesis. Less well studied pathways include the Wingless-type MMTV integration site (Wnt), Notch or mammalian target of rapamycin (mTOR) signalling which, nevertheless, take up relevant functions in cartilage and bone destruction, inflammation and angiogenesis (56-59).

[1] George Orwell, „Animal Farm" (1945)

In RA, the major pathways activated by TNFα are NF-κB and MAPK signalling (60). The sequential phosphorylation of MAPK kinases downstream of TNFα culminates in the activation of p38 and Jun kinase (JNK) and the expression of their target genes (60). The JNK-mediated activation of the AP-1 transcription factor (*via* phosphorylation of c-jun) is of particular importance for the transcriptional activation of MMPs and therefore joint destruction in RA (61).

The family of NF-κB proteins comprises five members (p65, c-Rel, RelB, p100/p52 and p105/p50) that form homo- or heterodimers binding to κB sites in regulatory sequences of genes (62). They are retained in the cytoplasm by Inhibitor of κB (IκB) proteins that mask their nuclear localisation sequence. Only upon phosphorylation by the IκB kinase (IKK) complex and the subsequent proteasomal degradation of the IκB proteins may NF-κB dimers relocate to the nucleus and regulate gene expression (62). Simplified, receptor ligation leads to a series of intracellular events involving different adapter proteins, ubiquitin ligases (e.g. the TNF receptor-associated factors [TRAFs]) and kinases (e.g. receptor interacting protein 1 [RIP1]) that result in the activation of the IKK complex which consists of the catalytic subunits IKK1 and IKK2 and the regulatory subunit NF-κB essential modulator (NEMO). The canonical pathway (induced among others by TNFα) depends on NEMO and IKK2 whereas the noncanonical branch (activated through RANK, for instance) requires the NF-κB-inducing kinase (NIK)-mediated phosphorylation of IKK1 (62, 63). The importance of TNFα and NF-κB signalling in the pathogenesis of RA is not only exemplified by the success of TNF-blocking biologicals in clinical practice (17) but also by the striking phenotype of the human TNF transgenic (hTNF tg) mice which spontaneously develop all histological signs of human RA (64).

Fundamentally, cells and organisms require means of closely controlling and terminating signal transduction to prevent a pathological activation of gene expression; in the case of NF-κB signalling these include TNFAIP3 (also known as A20), A20-binding inhibitors of NF-κB (ABINs), cylindromatosis protein (CYLD) and TRAF1 (63). Whereas TNFAIP3 and CYLD are deubiquitinating enzymes disrupting protein-protein interactions TRAF1 most likely inhibits NF-κB signalling through its lack of the ubiquitinating activity and competition with the other – NF-κB-activating - TRAF proteins (63). TNFAIP3 is of particular interest in RA since a single nucleotide polymorphism (SNP) in the *TNFAIP3* gene region is associated with RA, as determined by genome-wide association studies (65). Furthermore, experimental work convincingly suggests a functional role for TNFAIP3 in RA pathogenesis; mice with a knockout of TNFAIP3 specifically in the myeloid lineage develop a destructive arthritis (66) and treatment by adenoviral delivery of TNFAIP3 improves clinical signs of collagen-induced arthritis (CIA) in mice (67) underlining the important role of NF-κB activity in RA.

SF, as mesenchymal cells involved in cartilage turnover, are not a classical source of cytokines. In RA, however, they produce high amounts of cytokines and chemokines, especially when stimulated with proinflammatory mediators (19, 39) (also see Table 2). One such strong stimulus is TNFα; TNFα

stimulation activates NF-κB and MAPK pathways in RASF and leads to the downstream activation of matrix-degrading enzymes as well as cytokines and chemokines. These molecules then may further contribute to joint inflammation by the attraction (e.g. IL-8, MCP-1, RANTES) and activation of immune cells (e.g. IL-6) and, thus, SF are crucial players in the cytokine networks in RA.

Taken together, the networks of cytokines and signal transduction pathways among which TNFα and NF-κB signalling take up a central position enable synovial cells to communicate and coordinate their actions. Together with additional direct cell-cell contacts as well as the intrinsic activation of articular cells, in particular the SF, these networks are the basis for the continuing joint inflammation and destruction in RA.

2. Epigenetics

The concept of epigenetics was first formulated by developmental biologist Conrad Waddington and published in 1957 (68); for him, epigenetics was the study of how developmental decisions are reached, i.e. how the genotype gives rise to the phenotype (68, 69). Much later, in the 1990s, this perception of epigenetics was considerably changed and defined as the study of heritable alterations in gene expression that are not caused by changes in the underlying DNA sequence (i.e. genetics) (69). Nowadays, the somewhat expanded definition also includes transient gene expression changes that are only in theory heritable and, thus, should rather be understood as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (69).

As early as 1982, the first abnormally changed epigenetic modification in cancer was described showing a loss of DNA methylation in certain genomic regions (70). Since then, a tremendous progress has been made in understanding epigenetic regulation in development and disease. Especially during the last ten years, the field of epigenetics has experienced a burst. We know now four different modifications of DNA and 16 different histone modification classes (summarised in Table 3) (71) that are transferred by chromatin "writers" and removed by chromatin "erasers". These modifications can directly change chromatin structure or recruit proteins specifically recognising them, i.e. chromatin readers, which themselves bind additional chromatin modifiers and remodelling enzymes. The crosstalk of all epigenetic modifications at a genomic locus eventually influences the output of all DNA-based processes – DNA replication, repair and transcription (71).

A recent development in epigenetic research is the acknowledgement of the role of long non-coding (lnc) RNAs (RNAs more than 200 nt in length) in regulating chromatin structure. Whereas a few *cis*-acting lncRNAs (such as the XIST RNA involved in X chromosome inactivation) have been long known the first report of a *trans*-acting lncRNA was only published in 2007 in *Cell* (72). High-throughput genome-wide transcriptomic analyses have revealed the existence of tens of thousands of such lncRNAs and it becomes increasingly clear that these may play significant roles as signals, decoys, guides and scaffolds in transcriptional and epigenetic regulation (73). Thus, lncRNAs add yet

Table 3 Chromatin modifications, readers and their function (from Dawson MA & Kouzarides T (71))

Chromatin Modification	Nomenclature	Chromatin-Reader Motif	Attributed Function
DNA Modifications			
5-methylcytosine	5mC	MBD domain	transcription
5-hydroxymethylcytosine	5hmC	unknown	transcription
5-formylcytosine	5fC	unknown	unknown
5-carboxylcytosine	5caC	unknown	unknown
Histone Modifications			
Acetylation	K-ac	BromodomainTandem, PHD fingers	transcription, repair, replication, and condensation
Methylation (lysine)	K-me1, K-me2, K-me3	Chromodomain, Tudor domain, MBT domain, PWWP domain, PHD fingers, WD40/β propeller	transcription and repair
Methylation (arginine)	R-me1, R-me2s, R-me2a	Tudor domain	transcription
Phosphorylation (serine and threonine)	S-ph, T-ph	14-3-3, BRCT	transcription, repair, and condensation
Phosphorylation (tyrosine)	Y-ph	SH2[a]	transcription and repair
Ubiquitylation	K-ub	UIM, IUIM	transcription and repair
Sumoylation	K-su	SIM[a]	transcription and repair
ADP ribosylation	E-ar	Macro domain, PBZ domain	transcription and repair
Deimination	R→Cit	unknown	transcription and decondensation
Proline isomerisation	P-cis⇔P-trans	unknown	transcription
Crotonylation	K-cr	unknown	transcription
Propionylation	K-pr	unknown	unknown
Butyrylation	K-bu	unknown	unknown
Formylation	K-fo	unknown	unknown
Hyroxylation	Y-oh	unknown	unknown
O-GlcNAcylation (serine and threonine)	S-GlcNAc; T-GlcNAc	unknown	transcription

Modifications: me1, monomethylation; me2, dimethylation; me3, trimethylation; me2s, symmetrical dimethylation; me2a, asymmetrical dimethylation; and Cit, citrulline. Reader domains: MBD, methyl-CpG-binding domain; PHD, plant homeodomain; MBT, malignant brain tumor domain; PWWP, proline-tryptophan-tryptophan-proline domain; BRCT, BRCA1 C terminus domain; UIM, ubiquitin interaction motif; IUIM, inverted ubiquitin interaction motif; SIM, sumo interaction motif; and PBZ, poly ADP-ribose binding zinc finger.
[a]These are established binding modules for the posttranslational modification; however, binding to modified histones has not been firmly established.

another level of complexity to the epigenetic code which we are - despite all the progress made - only beginning to understand.

Histone modifications – a code for accessing the DNA

When outstretched the genomic DNA of a single cell is approximately two metres in length. To fit into the nucleus it must be compacted, which at the first level is achieved by the spooling onto histones to form the nucleosome, the basic functional unit of chromatin. One nucleosome is made up of a histone octamer (one H3:H4 tetramer and two H2A:H2B dimers of the core histones) around which 147 bp of DNA are wrapped (1.65 turns). The linker histone H1 binds to the nucleosome-free region between two neighbouring nucleosomes (20-50bp of linker DNA) and functions as a kind of molecular glue fixing the nucleosome position (74). Posttranslational modifications (PTMs) of histones occur mostly at the N-terminal tails protruding from the nucleosome and the most thoroughly studied modifications are acetylation, methylation and phosphorylation (Figure 4).

N^{ε}-acetylation of lysine (K) residues (transferred by histone acetyltransferases – KATs – and removed by histone deacetylases – HDACs) is universally connected with gene activation being wide-

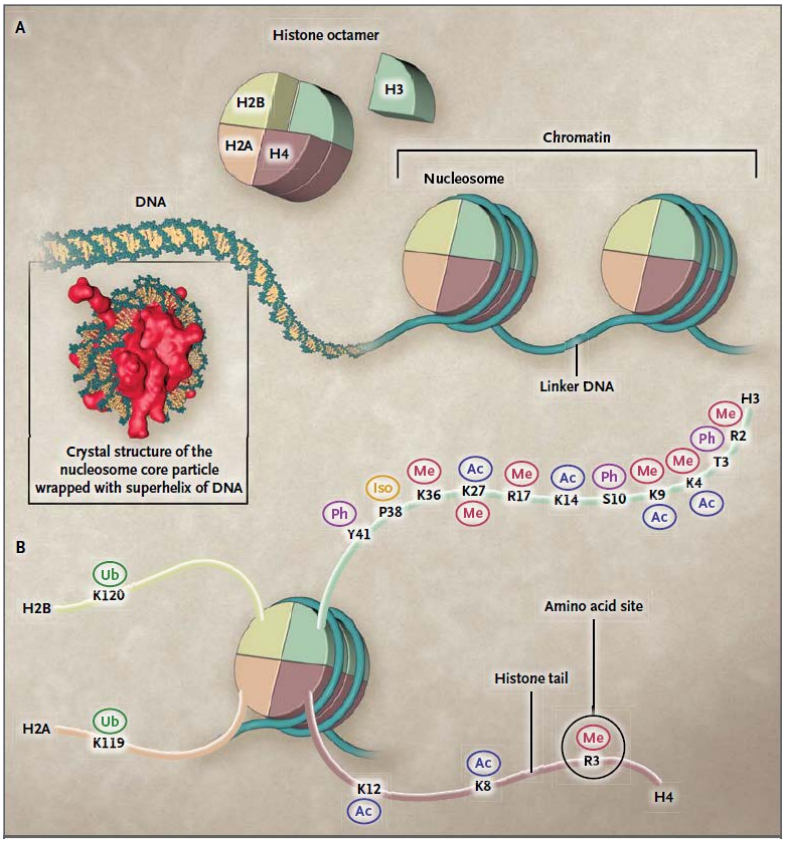

Figure 4 The nucleosome (from Dawson MA, Kouzarides T and Huntley BJP (75))

A. DNA is wrapped around the histone octamer consisting of two each of the four different core histones H2A, H2B, H3 and H4. B. Posttranslational modifications of the histone tails include but are not limited to methylation (Me), acetylation (Ac), phosphorylation (Ph) and ubiquitination (Ub).

ly found at promoters, enhancers and sometimes even throughout the whole region of actively transcribed genes (71). Neutralisation of the positively charged ε-amino group by acetylation loosens the interaction with the negatively charged sugar phosphate backbone of the DNA and, thus, histone acetylation-marked chromatin is considered to have a more "open" conformation facilitating the access of the transcriptional machinery. Additionally, histone acetylation is recognised by chromatin readers of the bromodomain and plant homeodomain (PHD) classes that effect epigenetic regulation or may recruit further epigenetic effectors (75).

Histone methylation occurs at lysine, arginine and (though less well understood) histidine residues; lysines are symmetrically mono-, di- and trimethylated at their ε-amino group while arginines are mono- or dimethylated (symmetrically or non-symmetrically) at their guanidino group. In contrast to (de)acetylases, the enzymes that write and erase histone methylation act in a site-specific manner. The best studied histone methylation marks are those at lysine side chains which are transferred by SET domain-containing histone methyltransferases (KMT) (with one exception) and removed by histone demethylases of the LSD1 or Jumonji family classes (71). Site-specific histone lysine methylation is connected to distinct transcriptional outputs; in this regard, histone 3 lysine 4 (H3K4), H3K36 and H3K79 methylation are associated with euchromatic regions of active gene transcription whereas H3K9, H3K27 and H4K20 methylation are found in transcriptionally silent,

heterochromatic regions (71, 74). Interestingly, H3K4 and H3K27 methylation may simultaneously be present in bivalent domains, especially in developmental genes, allowing for rapid gene activation or deactivation during development (76, 77). Methylation does not change the charge of amino acid residues within the histone tails and, thus, the mode of action of this modification depends on chromatin readers. Proteins with binding domains of the PHD finger and Royal Family (chromo, malignant brain tumour and tudor domain) then further execute the command of gene silencing or activation (71, 75).

Kinases, usually with already established roles in cell signalling such as JAK2, ATM and PIM1, can phosphorylate histone serines, threonines and tyrosines. By altering the charge of the histone, phosphorylation influences transcriptional regulation and chromatin condensation (71). Currently, there is not much knowledge about histone phosphatases although the very dynamic nature of histone phosphorylation clearly argues for their existence and importance (71).

Of interest, histone-modifying enzymes can additionally have non-histone protein targets (e.g. p53) whose activity they modulate which, when studying these enzymes, may cause problems in dissecting which of the effects seen are truly epigenetic in nature (71).

In conclusion, a picture emerges in which all histone modifications in combination regulate gene transcription, DNA repair and replication. Some modifications may act synergistically, some are mutually exclusive and yet others set chromatin into a state poised for further activation or silencing. This combinatorial action of the histone PTMs has been termed the "histone code" (78).

DNA methylation – marking DNA for transcriptional repression

DNA methylation, the methylation of cytosine residues at the fifth carbon atom of the pyrimidine ring (5mC), results in the repression of gene transcription. It almost exclusively occurs in the context of CpG dinucleotides within CpG islands (stretches of DNA longer than 200 bp and enriched for CpGs) which are present in ~70% of all promoter regions (71). These, however, are generally unmethylated and only acquire methylation during development and disease in a tissue-specific fashion (74). 5mC can directly inhibit transcription by preventing the binding of transcriptional activators or by the recruitment of methyl-CpG-binding domain (MBD) proteins that subsequently guide histone modifiers and chromatin remodellers towards the DNA (74). Recent genome-wide sequencing platforms have identified alterations in the methylation of CpG shores, regions less proximal to the promoter with a lower CpG density than CpG islands; for the moment, however, their role remains insufficiently understood (74, 79).

DNA methylation is carried out by the family of DNA methyltransferases (DNMTs). DNMT1 is the maintenance DNMT that re-establishes complete methylation at hemimethylated DNA during replication. The *de novo* DNMTs 3A and 3B establish new patterns of DNA methylation particularly during embryogenesis. A final prove for active DNA demethylation has not been found, yet. It is

assumed that the other covalent DNA modifications - 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine (cf. Table 3) – are intermediates of a step-wise oxidation by TET family 5-methylcytosine hydroxylases which eventually results in DNA demethylation (71). The existence of *bona fide* DNA demethylases is still an open question.

Chromatin remodelling – rolling the nucleosomes

The position of the nucleosomes on the DNA governs the accessibility of the transcription start site. Therefore, chromatin readers associate with the macromolecular machines of chromatin remodellers that move, destabilise, eject and restructure nucleosomes to enable or prevent transcription initiation, a process which is strictly ATP-dependent (74). In mammals, four families of chromatin remodelling complexes with different activities, functions and domain structures are known: the switching defective/sucrose nonfermenting (SWI/SNF), the imitation SWI (ISWI), the nucleosome remodelling and deacetylation (NuRD)/Mi-2/chromodomain helicase DNA-binding (CHD), and the inositol requiring 80 (INO80) families (71). The exact mode of how nucleosomes are positioned is not clear yet but it seems that the DNA sequence itself (i.e. preferred or disfavoured DNA motifs), DNA methylation, histone modifications and histone variants guide this process (80). Histone variants may greatly (e.g. H2A.Z, centromere protein A [CENP-A]) or only slightly (e.g. H3.3) deviate from canonical histones at the level of their amino acid sequence or domain structure. They display a specific spatial and/or temporal pattern of incorporation into the chromatin and thereby take influence on certain biological processes such as DNA replication (CENP-A instead of H3) or DNA repair (H2A.X) (80).

Epigenetics in disease – the cellular memory out of control

The collectivity of all epigenetic modifications (their presence as well as their absence) – the "epigenetic landscape" – controls the assembly of functional complexes impacting on all DNA-based processes. Not surprisingly, disruption of this tightly regulated system results in developmental defects and diseases. Aberrant epigenetic regulation has been found in multiple human diseases, first of all in cancer but also in neurodevelopmental disorders, neurodegenerative and neurological diseases, and autoimmune diseases (74).

Pathological epigenetics are mostly studied in the field of cancer research. Erroneous overexpression or silencing of epigenetic regulators causes significant changes in the epigenetic profiles of affected cells and thereby gene expression patterns are disturbed. In cancer, one generally finds global hypomethylation of DNA with hypermethylation at specific genomic loci, in particular promoter CpGs that should normally be unmethylated (79). This leads to the unwanted silencing of tumour suppressor genes which abets malignant transformation. Other promoters become

hypomethylated which enables the expression of oncogenes. Furthermore, global DNA hypomethylation is associated with the re-activation of transposable elements such as LINE1 which results in chromosomal instability (74). The global profile of histone modifications in cancer cells is skewed towards reduced acetylation, loss of the permissive H3K4me3 and the repressive H4K20me3 as well as increases in the repressive H3K9me3 and H3K27me3 marks (74).

In autoimmune rheumatic diseases, epigenetic changes have mainly been studied in systemic lupus erythematosus (SLE) and RA. DNA in T cells from SLE patients, for instance, is globally hypomethylated and several T cell function-related genes were shown to be overexpressed as a result (e.g. CD70, CD40 ligand and perforin 1) (81).

Recently it has become clear that not only the expression of epigenetic enzymes may be dysregulated but that genomic translocations and coding mutations of epigenetic regulators can form the basis of some diseases through a combination of genetic and epigenetic factors (79). One such example is the histone methyltransferase Enhancer of Zeste homologue 2 (EZH2): several gain-of-function mutations of this enzyme have been found in lymphomas that change the activity of EZH2 and, thus, may influence histone methylation patterns (82, 83). EZH2 was among the first epigenetic enzymes to be found overexpressed in a variety of malignant diseases; by its methylating activity on H3K27 EZH2 silences tumour suppressor genes (84). For this reason it has been considered as a *bona fide* oncogene for a long time. New data revealing a possible tumour suppressive function of EZH2 in certain haematologic malignancies have recently questioned this notion and further work is necessary to enlighten the ambiguous role of EZH2 in tumour biology (71, 85). Nevertheless, in all solid tumours studied EZH2 has been found overexpressed. EZH2 is a crucial regulator of developmental genes such as its primordial target, the homeobox transcription factor (HOX) gene family, and others of the Notch, Wnt and Hedgehog signalling pathways (86, 87). Interestingly these pathways are also implicated in the pathogenesis of RA.

The therapeutic interference with pathological epigenetic changes is a fascinating concept currently pursued by several investigators at various preclinical and clinical stages (71). Whereas inhibitors for enzymes modifying histone methylation (e.g. EZH2) and bromodomain chromatin readers are only in development in pre-clinical stages some epigenetic therapies have already received approval by the US Food and Drug Administration (FDA) (71). The hypomethylating agents azacitidine and decitabine, inhibitors of DNMTs, are in clinical use for myelodisplastic syndrome. Vorinostat and romidepsin are both pan-HDAC inhibitors showing great efficacy in patients with cutaneous T cell lymphoma (71, 79). New approaches in this field will be the combination of different epigenetic drugs to improve efficacy and reduce unwanted effects. Furthermore, it has been suggested to use epigenetic drugs in conjunction with conventional chemotherapy to better manage resistance to cancer therapy (79). In summary, this field holds excellent promise for the future improvement and replacement of current therapies to the benefit of the patient.

Rheumatoid arthritis – inflammatory epigenetics (?)

The first report of an aberrant epigenetic modification in RA goes back to the year 1990: Bruce Richardson and colleagues detected impaired DNA methylation in T cells from RA patients (88). Later it was found that MTX treatment could reverse this global loss of DNA methylation in peripheral blood mononuclear cells (PBMCs) (89). Detailed molecular studies into the pathogenetic function of DNA hypomethylation in RA, however, were not undertaken until the new millennium.

With respect to the fact that RA synovial cells appear to be defective in apoptosis Takami *et al.* demonstrated high DNA methylation levels in the promoter CpG island of the apoptosis-mediating receptor *DR3* gene compared with control OA synovial cells (90). RA peripheral blood lymphocytes presented with a loss of DNA methylation in the promoter region of the *EFNB1* gene and the associated overexpression of Ephrin B1 was connected to increased migration and cytokine production in these cells (91). Further studies identified DNA methylation defects in the promoter of *IL6* in RA PBMCs (loss of methylation at a single CpG site with concomitant IL6 upregulation (92)), the promoter of *FOXP3* in RA $CD4^+$ T cells (hypermethylation albeit without accompanying gene expression change (93)) and the X-linked *CD40L* promoter in female RA $CD4^+$ T cells (94). These data strongly argue for a pathogenetic role of DNA methylation in RA.

Epigenetic dysregulation represents an attractive and very likely explanation for the activated phenotype of RASF. In 2009, Karouzakis and co-authors demonstrated global DNA hypomethylation in RA synovial tissue and in RASF compared with OA samples alongside reduced DNMT1 levels in proliferating SF (95). Bisulfite sequencing revealed hypomethylation of the LINE1 transposon which is re-activated in RA. *In vitro*-hypomethylation of normal SF with 5-azacytidine upregulated the expression of genes involved in the pathogenesis of RA such as interleukins, adhesion molecules and matrix-degrading enzymes. These data suggested that the intrinsically activated phenotype of RASF is caused by epigenetic dysregulation, in particular by genomic hypomethylation (95). Follow-up projects identified reduced DNA methylation at the *CXCL12* gene and a concomitant overexpression of the CXCL12 chemokine in RASF (96) as well as reduced levels of the methyl donor S-adenosyl methionine (SAM). The latter finding was explained by the increased recycling of polyamines in RASF posing the possibility of global hypomethylation being, at least in part, due to the consumption of SAM by this pathway (97). Studying the methylome of RASF and OASF, 1859 differentially methylated loci were recently identified many of which were connected to genes involved in joint architecture and inflammation, for instance integrins and signal transduction pathway components (e.g. STAT3) (98).

Despite the relative wealth of information on DNA methylation detailed analyses of other epigenetic modifications in RA are rather scarce. Most studies have concentrated on the use of HDAC inhibitors (HDACi) in preclinical models of the disease and the expression pattern of histone acetylation-modifying enzymes is still a matter of controversy. Huber *et al*. reported the HDAC/KAT

activity ratio to be decreased in RA synovial tissue together with reduced protein levels of HDAC1 and 2 (99). This was later contradicted by a study from Kawabata and co-workers; they found increased HDAC activity and increased HDAC1 expression in RA synovial tissue (100). It was speculated that this discrepancy may have arisen from differences in therapy and the method of acquisition of the biological material (45, 100). Either way, more research is necessary to conclusively clarify this question. Looking at class III HDACs (sirtuins), SIRT1 was found to be overexpressed in RA synovial tissue, RASF and RA blood monocytes and to mediate apoptosis resistance in RASF as well as IL-6 and TNFα secretion from RASF and monocytes, respectively (101).

Experiments using various HDACi generally showed beneficial effects in RA animal models and in *in vitro* assays (reviewed in 102). Different HDACi, for instance, suppressed cytokine production and induced apoptosis in peripheral blood and synovial fluid Mϕ, and inhibited the production of cytokines from synovial tissue explant cultures (103). RA PBMCs, which displayed increased HDAC activity compared to healthy control PBMCs, were inhibited to secrete IL-6 by a no-

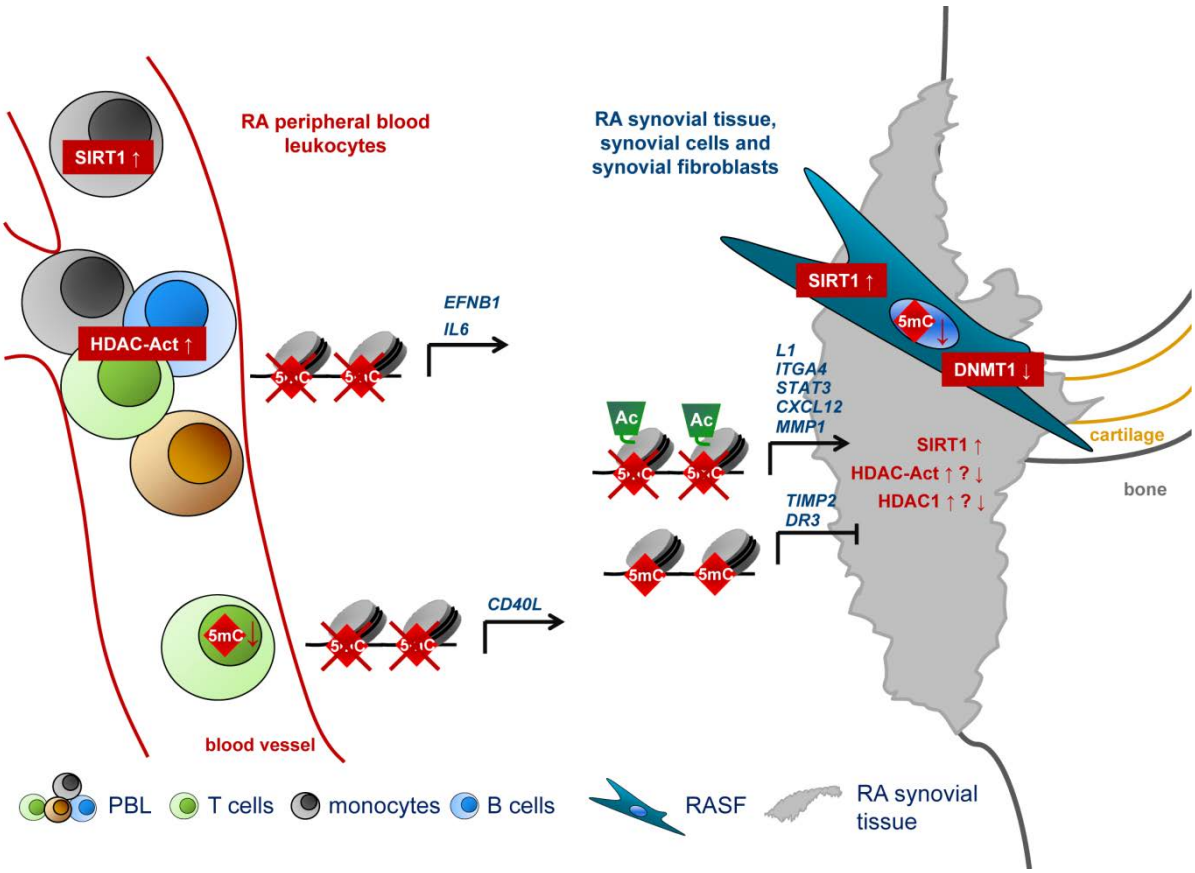

Figure 5 Epigenetic changes in RA

In RA, global as well as promoter DNA methylation and histone acetylation have been studied in peripheral blood leukoocytes (left), synovial tissue and synovial cells, in particular SF (right). Together, these epigenetic aberrations contribute to immune cell activation and migration, and SF activation ultimately favouring a synovial environment of chronic inflammation, hyperplasia and tissue destruction. Abbreviations: PBL peripheral blood lymphocytes, SIRT1 silent mating type information regulation 2 homologue (sirtuin) 1, HDAC histone deacetylase, Act activity, 5mC 5-methylcytosine, Ac histone acetylation, DNMT1 DNA methyltransferase 1, EFNB1 ephrin B1, IL6 interleukin 6, CD40L cluster of differentiation 40 ligand, L1 LINE1 retrotransposable element, ITGA4 integrin alpha 4, STAT3 signal transducer and activator of transcription 3, CXCL12 chemokine (C-X-C motif) ligand 12, MMP1 matrix metalloprotease 1, TIMP2 tissue inhibitor of metalloproteases 2, DR3 death receptor 3.

vel HDAC3-specific HDACi (104). HDACi studies, however, only give indirect evidence of epigenetic changes in RA. Considering the many non-histone protein targets of histone modifying enzymes the effects observed using HDCAi may after all not be related to epigenetic mechanisms. In line with this, Grabiec *et al.* demonstrated that HDACi-mediated IL6 repression in RASF was not due to epigenetic regulation but due to changes in NF-κB activity and IL6 mRNA stability (105).

Using chromatin immunoprecipitation Maciejewska-Rodrigues and co-workers were able to show increased H4 acetylation levels at the *MMP1* promoter in RASF as compared to OASF which may lead to the upregulation of MMP1 in RASF (106). Another study implicates increased H3 acetylation at the IL17 promoter in synovial Th17 differentiation (107).

In summary, several lines of evidence point towards the involvement of epigenetic dysregulation in the pathogenesis of RA (Figure 5). Still, there is a vast knowledge gap to bridge until the epigenetics of the rheumatoid joint are understood. In particular, the exact effects of HDACi on histone and non-histone targets must be distinguished and histone modifications other than histone acetylation have not been addressed at all so far.

3. microRNAs

Tiny finetuners of gene expression under strict control

microRNAs (miRNAs) are approximately 22nt-short non-coding RNAs that regulate the expression of protein-coding genes at the posttranscriptional level by degradation and/or inhibiting translation of the target mRNA. The first miRNA (*lin-4*) was discovered in 1993 in the nematode *Caenorhabditis elegans* providing the first explanation how the non-coding *lin-4* gene product could repress the expression of the LIN-14 protein without obvious changes in the expression of the corresponding mRNA (108).

Since the discovery of the founding member *lin-4*, thousands of other, generally evolutionarily conserved, miRNAs have been found across the whole metazoan kingdom as well as in plants. Plant and animal miRNAs, however, differ in several aspects of their biogenesis and mode of action implying independent evolution of miRNA pathways in the two kingdoms (108). The broad conservation of miRNAs and miRNA pathways within Animalia and Plantae, nevertheless, argues for the importance of this class of gene expression regulators.

Small non-coding RNAs such as transfer RNAs (tRNAs) and small nuclear RNAs (snRNAs) are essential components of the cellular translation and splicing machineries and are, as such, transcribed as housekeeping genes from RNA polymerase III promoters. In contrast, miRNAs are just as protein-coding genes transcribed by RNA polymerase II and, thus, miRNA expression is subject to similar mechanisms of gene regulation (109). miRNAs are encoded by independent genes or may be derived from introns of mRNAs after the splicing of the primary transcript. In the canonical miRNA biogene-

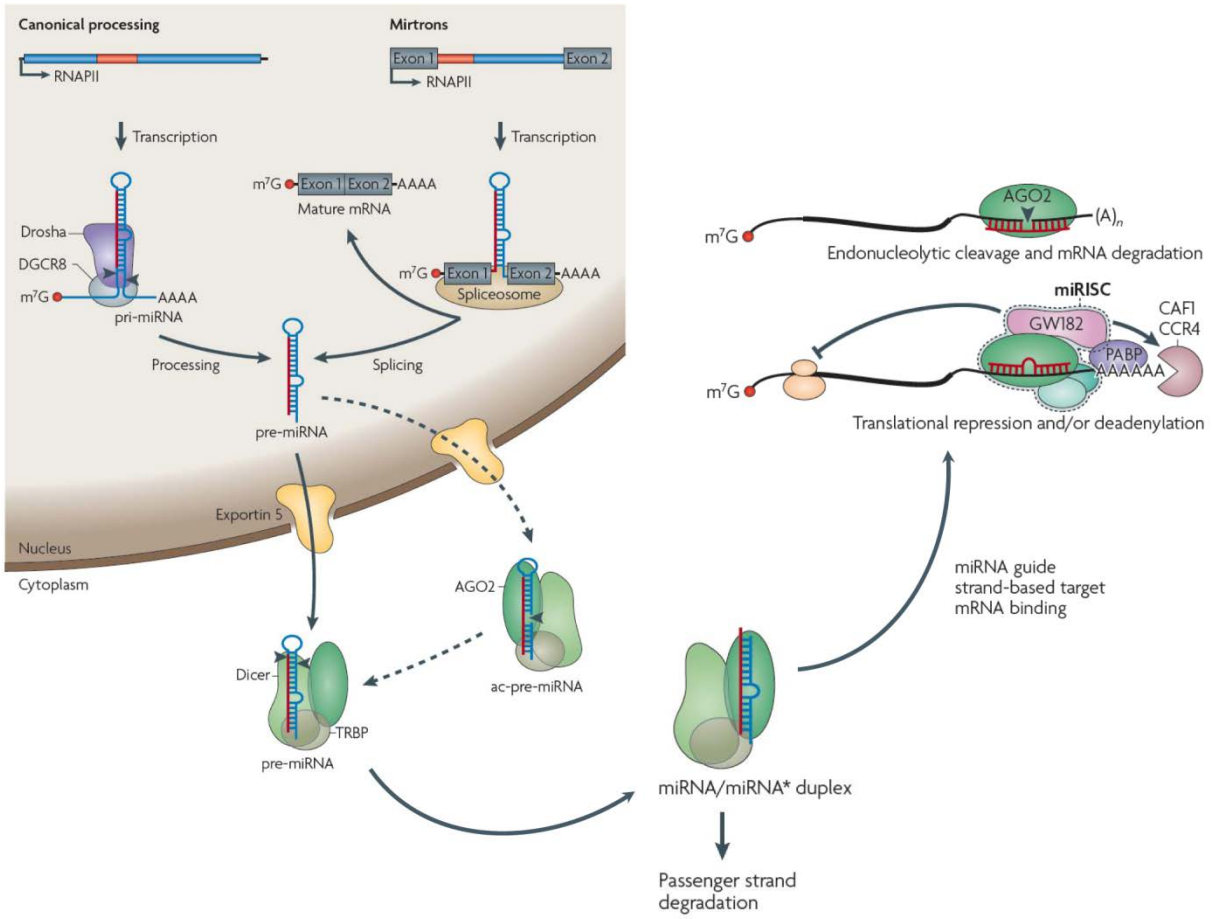

Figure 6 microRNA biogenesis (adapted from Krol J, Loedige I and Filipowicz W (109))

MicroRNA genes are transcribed by RNA polymerase II, processed by the RNase III enzyme Drosha or alternatively the spliceosome (mirtrons), exported from the nucleus by Exportin 5 and cleaved by a second class III-RNase, Dicer. The mature miRNA guide strand is loaded onto the miRNA-induced silencing complex (miRISC) and executes miRNA functions while the passenger strand is (usually) degraded. Abbreviations: RNAPII RNA polymerase II, DGCR8 DiGeorge syndrome critical region 8, pri-miRNA primary miRNA, pre-miRNA precursor miRNA, m^7G 5'7-methylguanosine cap, AGO2 argonaute 2, ac-pre-miRNA AGO2-cleaved pre-miRNA, TRBP transactivation-responsive RNA-binding protein, GW182 glycine-tryptophan protein of 182 kDa, PABP poly(A)-binding protein, CCR4 CC chemokine receptor type 4, CAF1 CCR4-associated factor 1

sis pathway (Figure 6), these primary transcripts (pri-miRNA) fold into hairpin structures that are recognised by the microprocessor complex whose core components are the RNA-binding protein (RBP) DiGeorge syndrome critical region 8 (DGCR8) and the RNase III enzyme Drosha (109, 110). The microprocessor cleaves the hairpin at its base to release the ~70nt precursor- (pre)-miRNA which is bound by Exportin 5-Ran and is actively transported to the cytoplasm with the help of Ran-mediated GTP hydrolysis. There the hairpin is cleaved off by the RNase III Dicer to yield an unstable duplex of the mature miRNA guide strand and passenger strand (miRNA*) with 2nt of 3' overhang in both strands. The transactivation-responsive RBP (TRBP) supports the Dicer-mediated pre-miRNA cleavage and then ensures the loading of the guide strand into an argonaute (Ago) protein and assembly of the miRNA-induced silencing complex (miRISC) (110). Selection of guide and passenger strand is dependent on the thermodynamic stability of the miRNA/miRNA* duplex and the passenger

strand normally is degraded (109). Recently, however, evidence for stable expression and biological functions of miRNA* species has been accumulating (109).

Alternative pathways for the biogenesis of miRNAs include the mirtron pathway (Figure 6), small nucleolar (sno) RNA-derived miRNAs, tRNAse Z-derived miRNAs and the special case of Dicer-independent miR-451 biogenesis (111). Mirtons are very short introns in which the spliceosome substitutes for the necessity of the microprocessor complex. After debranching of the resulting lariat structure the mirtron product resembles a pre-miRNA and further proceeds through the canonical pathway (111). pri-miR-451, in contrast, initially goes through the canonical steps of Drosha-mediated cleavage and export from the nucleus. But pre-miR-451 cannot be cut by Dicer and instead may be loaded onto Ago2 where it is sliced to yield the Ago2-cleaved (ac-)pre-miR-451 which is further processed to become the mature miR-451 by a nuclease yet to be identified (111).

miRNA biogenesis is tightly controlled at basically every level of the pathway to ensure correct spatiotemporal miRNA expression (109, 112). Being RNA polymerase II-dependent, pri-miRNA transcription is under the control of transcription factors and regulatory elements as well as epigenetic mechanisms. Therefore, miRNA promoters closely resemble the promoters of protein-coding genes and, similarly, their activity is regulated in a tissue-, development- and stimulation-dependent manner (109, 112). The further processing steps are greatly influenced by RBPs and other accessory proteins. Cellular total levels of Drosha, DGCR8 and Dicer as well as the activity of these components are rigorously regulated. The DEAD box helicases p68 (DDX5) and p72 (DDX17), for instance, are essential elements of the microprocessor and knockout of either one causes embryonic lethality in mice (112). Another example is the alternative splicing factor heterogeneous nuclear ribonucleoprotein (hnRNP) A1 which was shown to enhance pre-miR-18a maturation without influencing the other miRNAs derived from the polycistronic miR-17-92 cluster (encoding miR-18a and five other miRNAs). Other levels of regulation may be RNA editing by adenosine deaminases that act on RNA (ADARs) which influences the processing and the control of nuclear export of the pre-miRNA (109, 112). All these means of regulating miRNA biogenesis may be carried out in a cell type-, stimulation-, tissue-dependent fashion and are in part miRNA-specific; therefore, they may account for differences observed in individual miRNA levels (109, 110).

miRNAs bind their target mRNAs through perfect complementarity (especially in plants) or, much more often, partial complementarity with the 3’ untranslated region (UTR). The binding part of the miRNA is called the seed region and generally constitutes nucleotides 2 to 7 (or 8) at the 5’ end of the mature miRNA (Figure 7A, B). Since binding specificity is determined by only six or seven nucleotides it is not surprising that one miRNA can have several hundreds of targets. At the same time, one mRNA may be the target of multiple miRNAs. Computational prediction programmes avail of the sequence-specific nature of miRNA-mRNA interactions to predict potential miRNA target genes which substantially eases the search for biological functions of miRNAs. Nevertheless, these

algorithms produce a high proportion of false positive predictions while missing out other targets so that direct miRNA-target interactions still need to be verified for each pair individually (113, 114).

Upon maturation miRNAs are incorporated into the miRISC complex which is facilitated by the interaction of Dicer with several other proteins forming the RISC loading complex (112). The major components of miRISC are one of the four Ago proteins and the glycine-tryptophan repeat-containing protein of 182 kDa (GW182). The exact mode of action of the miRISC has not been elucidated so far but it appears that miRISC inhibits translation initiation and possibly elongation and mediates mRNA deadenylation, decapping and degradation (115) (Figure 7C). In eukaryotes, translation starts with the recruitment of the translation initiation factor eIF4F to the 5'cap of the mRNA and the subsequent assembly of the 43S pre-initiation complex (consisting of the 40S small subunit of the ribosome, the initiator tRNA and a couple of initiation factors) (116). This complex then starts scanning the mRNA in 5'→3' direction and upon base pairing of the anti-codon with the start codon all initiation factors

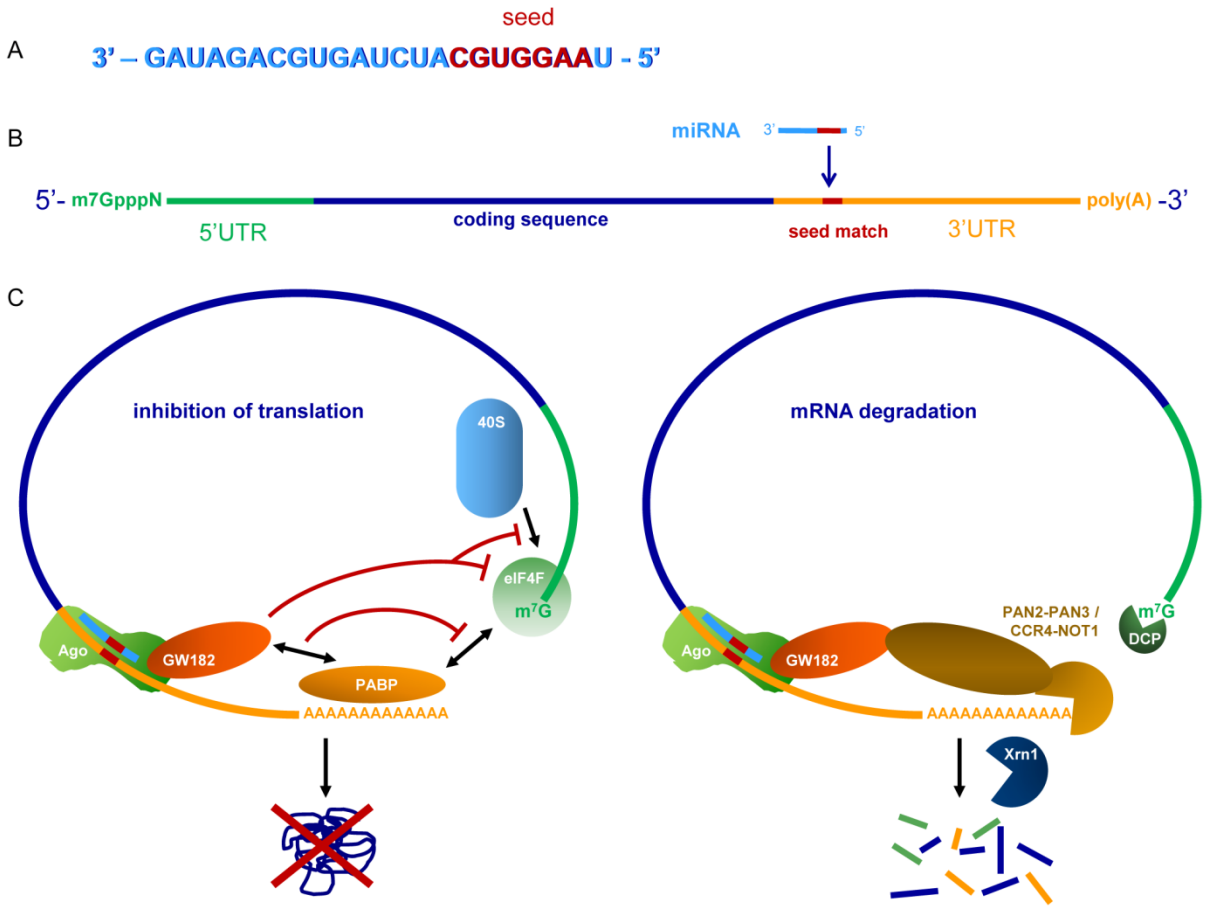

Figure 7 Mechanisms of miRNA-mediated gene repression

A. Sequence of a mature miRNA (miR-18a) with the seed sequence in dark red. B. Structure of a typical mRNA consisting of the 5' untranslated region (UTR) (dark green) with the 5'cap, coding sequence (dark blue) and 3'UTR (orange) with the poly(A) tail. miRNA binding sites (the sequence reverse complementary to the seed sequence = seed match, dark red) usually lie in the 3'UTR. C. Upon miRISC assembly at the target mRNA the GW182-mediated protein-protein interactions inhibit mRNA circularisation and binding of the translation initiation factor eIF4F to the 5'cap thereby preventing 40S ribosomal subunit recruitment. GW182 recruits two deadenylase complexes, CCR4-NOT1 and PAN2-PAN3. Removal of the poly(A) tail favours the decapping of the mRNA (DCP1-DCP2 complex) and the subsequent 5'→3'-exonucleolytic breakdown of the mRNA (Xrn1).

are released and the large 60S ribosomal subunit is allowed to bind. Of importance, to increase the efficiency of translation, mRNAs are circularised by an interaction of the 5'cap binder eIF4F and the poly(A) binding protein (PABP) thereby placing the ribosome in an ideal position to re-initiate translation after a cycle has been finished (116). The miRISC is believed to prevent binding of the 5' cap of the mRNA by eIF4E (a subunit of eIF4F) thereby impeding ribosome assembly and the interaction of GW182 with the PABP might interfere with mRNA circularisation (115). As to miRNA-mediated mRNA decay, GW182 was shown to bind two deadenylase complexes, the CC chemokine receptor type 4 – negative regulator of transcription (CCR4-NOT) and the poly(A)-specific ribonuclease 2 & 3 (PAN2-PAN3) deadenylation complexes. In eukaryotes, removal of the poly(A) tails destabilises the mRNA and so, subsequently, the 5'cap of the mRNA is removed by a decapping enzyme complex (DCP1-DCP2), and the mRNA finally is broken down by the 5'-3' exoribonuclease 1 (Xrn1) (115). The relative proportion of translation inhibition and mRNA degradation in miRNA-mediated gene repression remains one of the most important issues that have not yet been entirely illuminated. Recent studies point towards a sequential model in which inhibition of translation precedes mRNA degradation and that the block in translation possibly favours deadenylation and the subsequent mRNA decay (115, 117). This model also allows for the possibility that gene repression takes place only at the protein level, i.e. it does not proceed through all silencing steps, e.g. to achieve better reversibility of the silencing (117).

Target recognition and accessibility is another tightly regulated level of the miRNA pathway. An array of RBPs binding to 3'UTRs can counteract or facilitate the miRNA-target interaction. The AU-rich element-binding Hu proteins are stabilisers of mRNAs; in this regard, the ubiquitously expressed HuR protein was shown to relieve miR-122-mediated repression of cationic amino acid transporter 1 mRNA by binding to its 3'UTR (110, 115). A hnRNP family member, hnRNPL, may prevent miRNA binding to the VEGFA mRNA during hypoxia and another RBP, dead end 1, rescues the cyclin-dependent kinase inhibitor 1B (CDKN1B, p27) from repression by direct interference with miRNA binding sites (110, 115). The PUF RNA binding protein Pumilio 1 (Pum1), in contrast, enables the binding of miR-221 and miR-222 to the CDKN1B mRNA upon growth factor stimulation by changing the secondary 3'UTR structure making it more accessible (110, 115). A new fascinating mechanism of controlling miRNA activity, and thus target binding, has recently emerged with the discovery of competing endogenous RNAs (ceRNAs), a class of lncRNA also termed natural miRNA sponges (118). Without having another obvious function these ceRNAs act as decoys for miRNAs thereby preventing miRNA binding to other target RNAs. A prime example is the PTEN pseudogene *PTENP1* which harbours several miRNA binding sites in the 5' region of its "3'UTR" that is highly similar to the PTEN 3'UTR. Therefore, PTENP1 acts as a tumour suppressor by intercepting miRNAs that are otherwise bound to repress the PTEN mRNA (119).

In some cells, also the subcellular localisation of miRNAs is under deliberate control, a mechanism that has been shown to be exceptionally important in nerve cells where miRNA and, thus,

target expression in the cell body and dendrites can differ substantially (109). The last instance of miRNA regulation is the stability of the miRNA itself. In general, miRNAs are highly stable molecules with half-lives of several hours or even days but under certain circumstances miRNA turnover needs to be speeded up and regulated miRNA decay has been found in multiple cell types and for various miRNAs (109).

In summary, miRNA biogenesis and action represent a highly concerted pathway whose detailed mechanisms are becoming gradually clear. Disruption of this pathway can have detrimental effects and dysregulated expression of miRNAs or miRNA pathway components is found in many human pathologies. As with epigenetics, miRNA dysregulation is most extensively studied in cancer research and numerous cancer-associated miRNAs ("oncomirs") have been identified. Moreover, miRNAs play significant roles in various other processes such as angiogenesis, haematopoiesis, vascular remodelling, cardiac hypertrophy, and myogenesis and, as a result, have been implicated in the pathogenesis of many diseases (120). Importantly, also biological samples from patients with autoimmune diseases such as SLE and RA display aberrant miRNA expression profiles and consequential functional abnormalities.

Micro-regulation of rheumatoid arthritis

The first paper to connect RA with the miRNA pathway did not deal with the expression of miRNAs but with autoantibodies to GW bodies (cytoplasmic foci to which the miRNA silencing machinery locates) in the sera of patients of different medical conditions (121). This study, however, included only three RA patients and all three had additional other diagnoses. The impact of this interesting but somewhat anecdotic finding on the pathogenesis of RA must therefore be questioned for the moment.

Nevertheless, intensive research over the last five years has produced a first comprehensive list of miRNAs dysregulated in RA (Table 4). The first study reporting miRNA differential expression in RA came from Stanczyk and colleagues (122). They showed upregulation of miR-155 in RASF and RA synovial tissue compared to OA samples. Additionally, miR-146a was found to be increased in RASF. Enforced expression of miR-155 in RASF reduced the expression of MMP1 and 3 and, thus, miR-155 upregulation was suggested to be a protective mechanism that counter regulates tissue destruction (122). Other miRNAs identified to be upregulated in RASF were miR-223, miR-142-5p and -3p, miR-133a (123), miR-221/222, miR-323 (124) and miR-203 (125) whereas miR-124a (123) and miR-34a* (126) were reduced. Individual miRNA expression differences were also found in whole synovial tissue and peripheral blood cells (see Table 4). Apart from evaluating human biological material some papers reported on the expression of miRNAs in animal models of RA. Thus, miR-155 (127) and miR-146a were shown to be increased in the articular tissue of mice with CIA whereas the expression of miR-23b was reduced (128). Paradoxically, intravenous injection of miR-146a inhibited

Table 4 Differential expression of miRNAs in human RA

miRNA change	cell type/biological sample[#]	function/effect of miRNA expression change	miRNA target[##]
miR-16 ↑	PBMCs (129) synovial fluid (130)		
miR-124a ↓	RASF (123)	RASF proliferation ↑, MCP-1 and Angiogenin release ↑ (123)	CDK2 MCP1 (ANG)
miR-132 ↑	PBMCs (129)		
miR-132 ↓	plasma (130)		
miR-133a ↑	RASF (123)		
miR-142-5p ↑	RASF (123)		
miR-142-3p ↑	RASF (123)		
miR-146a ↑	RASF (122, 123) CD4+ T cells (131) synovial tissue (128, 132) PBMCs (129) synovial fluid (130)	T cell apoptosis ↓ (131)	(FAF1)
miR-155 ↑	RASF (122, 124) synovial tissue (122) PBMCs (129) synovial fluid (130) synovial Mϕ (127)	MMP1 and MMP3 expression in RASF ↓(122) Mo secretion of TNFα, IL-6, IL-8, IL-1β ↑, of IL-10 ↓ (127)	SHIP1
miR-203 ↑	RASF (125)	MMP1 and IL6 expression in RASF ↑ (125)	
miR-221/222 ↑	RASF (124)		
miR-23b ↓	synovial tissue (128)	increased NF-κB signalling (128)	TAB3 IKKα TAB2
miR-223 ↑	RASF (123) T cells (133) synovial fluid (130) synovial tissue (134)	inhibits *in vitro* osteoclastogenesis (134)	
miR-323 ↑	RASF (124)	increased Wnt signalling (124)	(BTRC)
miR-34a* ↓	RASF (126)	RASF apoptosis resistance (126)	XIAP

[#]RA sample compared to OA or healthy control, [##]new miRNA targets identified, for genes in parentheses no direct miRNA-target interaction proven. CDK2 = cyclin-dependent kinase 2, MCP1 = monocyte chemoattractant protein 1, FAF1 = Fas-associated factor 1, SHIP1 = Src homology 2-containing phosphatase 1, TAB = TGF-β-activated kinase 1/MAP3K7 binding protein, IKKα = Inhibitor of κB kinase a, BTRC = β-transducin repeat containing, XIAP = X-linked inhibitor of apoptosis

bone destruction in the CIA model, an effect that was explained by the fact that TRAF6 and interleukin-1 receptor-associated kinase 1 (IRAK1), components of the NF-κB signalling cascade which is crucial for osteoclastogenesis, are direct targets of miR-146a (135). Analysing SF derived from the hTNF tg mouse model of arthritis, Pandis and co-workers found several differentially expressed miRNAs some of which were similarly dysregulated in human RASF (miR-155, miR-221/222, miR-323) (124). It is remarkable that miR-146a and miR-155 are overexpressed in every biological sample studied so far in the context of RA. Considering their important role in regulating immune function and inflammation (136, 137), however, this is not very surprising. miR-155, for instance, is critically involved in B cell maturation and B and T cell function and some of the validated targets include IKKε, suppressor of cytokine signalling 1 (SOCS1), and receptor-interacting

serine/threonine kinase 1 (RIPK1), all of them elements of inflammatory signalling pathways (136, 138). Beside IRAK1 and TRAF6, miR-146a was further demonstrated to target for example IRAK2, IL-8, Fas-associated via death domain (FADD), and Rho-associated coiled-coil containing protein kinase 1 (ROCK1) thereby affecting the innate and the adaptive immune response, cell survival and invasion (137).

With respect to miR-146a and its target IRAK1, Chatzikyriakidou *et al.* studied SNPs in both genes in the context of RA (139). Whereas the miR-146a variant did not predispose to develop RA one of the SNPs in the 3'UTR of IRAK1 was connected with susceptibility to RA (139). But since that SNP does not overlap with the miR-146a seed matches it remains to be determined which biological function it has and whether it might influence miRNA binding to the IRAK1 mRNA. Nevertheless, this study should arguably inspire more comprehensive analyses of disease pathology-relevant SNPs and their potential to interfere with the miRNA pathway.

Although the interest in measuring miRNA expression has been high in recent years studies associating miRNA dysregulation with an actual biological function and identifying new miRNA target genes relevant to the pathogenesis of RA have been rare. The most notable papers in this regard were published by Kurowska-Stolarska *et al.* in 2011 (127) and by Zhu and co-workers in 2012 (128). Src homology 2-containing inositol phosphatase-1 (SHIP1), an inhibitor of inflammatory signalling pathways, was identified as a direct target of miR-155 and to be downregulated in RA synovial tissue Mϕ (127). miR-155 knockout mice were protected against CIA as evidenced by lack of synovial inflammation and cartilage and bone destruction and this went along with increased expression of SHIP1 in bone marrow-derived Mϕ (127). A simultaneously published paper supports the data of miR-155$^{-/-}$-protection from CIA and shows that, in contrast to CIA, miR-155 knockout does not influence the development of K/BxN serum transfer arthritis although it could inhibit osteoclastogenesis (140). Zhu *et al.* found miR-23b to directly repress several genes involved in inflammatory signalling pathways, namely TGF-β-activated kinase 1/MAP3K7 binding protein (TAB) 2 and 3 and IKKα (128). Concomitant with reduced expression of miR-23b in RA synovial tissue the three miR-23b targets were upregulated at the protein level. In line with these findings, miR-23b was shown to suppress IL-17-induced NF-κB signalling and, thus, miR-23b silencing in RA was associated with increased inflammatory signalling (128). The authors further studied the role of miR-23b in CIA using miR-23b tg mice as well as adenoviral delivery of miR-23b and found that in both experimental settings CIA was suppressed altogether identifying miR-23b as a major inhibitor of inflammatory responses and potential therapeutic target in RA (128). In another paper, downregulation of miR-124a in RASF could be directly linked to the increased expression of MCP-1 and cyclin-dependent kinase (CDK) 2. Hence, miR-124a is involved in regulating the chemoattractive potential and proliferation of RASF (123). Finally, validating the X-linked inhibitor of apoptosis (XIAP) as a direct target of miR-34a*, Niederer *et al.* connected the silencing of miR-34a* in RASF with increased XIAP levels and, thus, the resistance to apoptosis inherent to RASF (126).

Summarising the above, there is ample evidence for miRNA dysregulation in RA. Considering their many targets and the somehow ambiguous roles they might assume (e.g. miR-146a, miR-155) the question of whether individual miRNAs are suitable targets for the treatment of RA persists. Also, many studies have for the moment merely concentrated on measuring miRNA levels without looking for biological functions. Therefore, further research is indispensable to better understand the miRNome of RA and determine its druggability.

4. Objectives

EZH2 – an oncogenic histone methyltranferase in rheumatoid arthritis epigenetics

RASF are key players in the pathogenesis of RA producing large amounts of matrix-degrading enzymes and inflammatory mediators thereby significantly contributing to chronic inflammation and joint destruction (19). RASF present with an activated phenotype for which the molecular basis still is incompletely understood. Epigenetic dysregulation offers an attractive explanation for the intrinsic activation of RASF (45). Whereas DNA methylation and histone acetylation have already been studied in the context of RA histone methylation has not been addressed yet. In an initial screen looking for the expression of different histone methyltransferases and demethylases EZH2 was found to be upregulated in RASF. With respect to the tumour-like phenotype of RASF this histone methyltransferase is of particular interest since it was found to be overexpressed in common solid tumours and therefore is considered as an oncogene participating in the epigenetic silencing of tumour suppressor genes (84, 85). In **Chapter II** of my doctoral thesis the data on the role of EZH2 in the activated phenotype of RASF are presented. Briefly, (i) the expression of EZH2 in RASF and (ii) the regulation of EZH2 by inflammatory cytokines were studied. Moreover, by experimentally manipulating EZH2 levels in RASF we (iii) identified an EZH2 target gene in RASF and (iv) validated constitutive epigenetic repression of that target gene in RASF utilising chromatin immunoprecipitation assays.

miR-17-92 – Oncomir-1 as a driving force of inflammation and matrix degradation

The miR-17-92 cluster – also termed *oncomir-1* due to the fact that it was the first miRNA cluster connected to the development of malignant tumours – assumes unique roles in development (e.g. the immune system, lung and cardiovascular system) and disease (e.g. cancer, autoimmunity) (141, 142). With our previous work we could show that miR-17-92, and more specifically, miR-17 and miR-20a, both derived from this cluster, are involved in vascular remodelling of small pulmonary arteries (143, 144). IL-6 could be demonstrated to upregulate the expression of miR-17-92 in pulmonary arterial endothelial cells and, through the action of miR-20a, to silence the bone

morphogenic protein receptor type II, loss of which is a hallmark of the pathogenesis of pulmonary arterial hypertension (143, 144). We could furthermore show that miR-18a from the miR-17-92 cluster is part of a positive feedback loop in IL-6-induced STAT3 signalling (145). Thus, based on the known role of miR-17-92 in cancer, autoimmunity and our own work in pulmonary hypertension, we speculated whether miR-17-92 could also be involved in the pathogenesis of RA and the aggressive phenotype of RASF. One of the most important cytokine pathways in RA is TNFα-induced NF-κB signalling and, thus, in **Chapter III** of my doctoral thesis we show that, by forming a positive feedback loop, miR-17-92 plays a similar role in TNFα-NF-κB-signalling as it does in IL-6-STAT3 signalling. To that purpose (i) the expression of miR-17-92 in RASF in response to TNFα was measured and the involvement of the NF-κB pathway in miR-17-92 regulation by TNFα was determined. (ii) Precursor molecules of individual miRNAs from the miR-17-92 cluster were transfected into RASF and the expression of matrix-degrading enzymes and inflammatory mediators was measured to determine the contribution of those miRNAs to the inflammatory and destructive gene expression signature of RASF. Using computational prediction programmes and reportergene assays (iii) a new direct target of miR-18a was identified and (iv) its involvement in TNFα-induced NF-κB signalling in RASF was further validated.

CHAPTER II

Expression and function of EZH2 in synovial fibroblasts: epigenetic regulation of the Wnt inhibitor SFRP1 in rheumatoid arthritis

Michelle Trenkmann, Matthias Brock, Renate E. Gay, Christoph Kolling, Rudolf Speich, Beat A. Michel, Steffen Gay, Lars C. Huber. *Ann Rheum Dis*. 2011 Aug; 70(8): 1482-8

1. Abstract

Objectives: To study expression, regulation and function of the histone methyltransferase EZH2 in synovial fibroblasts (SF) from patients with rheumatoid arthritis (RA) and osteoarthritis (OA).

Methods: SF were obtained from RA and OA patients undergoing joint surgery. Expression levels were assessed by quantitative real time PCR and Western blot. Kinase inhibitors and reporter gene assays were employed to study signalling pathways. Functional analyses included EZH2 overexpression by plasmid transfection and gene silencing by siRNA. Chromatin immunoprecipitation assay was used to analyse histone methylation within distinct promoter regions.

Results: By studying expression and function of EZH2 in SF we found that EZH2 is overexpressed in RASF and further induced by tumour necrosis factor alpha through the NF-κB and Jun kinase pathways. As a target gene of EZH2 we identified secreted frizzled related protein (SFRP)1, an inhibitor of Wnt signalling, which is associated with the activation of RASF, and show that SFRP1 expression correlates with the occupation of its promoter with activating and silencing histone marks.

Conclusions: Our data strongly suggest that the chronic inflammatory environment of the RA joint induces EZH2 and, thus, might cause changes in the epigenetic programmes of SF.

2. Introduction

Rheumatoid arthritis (RA) is an inflammatory disorder characterized by the progressive destruction of joints and bones. The pathogenesis of RA is still not completely understood so far; however, within the inflamed and hyperplastic synovium of RA patients, synovial fibroblasts (RASF) have been identified as important mediators that promote ongoing inflammation and destruction. These cells show an aggressive phenotype, which is characterized by the production of inflammatory mediators and matrix-degrading enzymes, activation of signalling pathways, and by the dysregulation of oncogenes and tumour suppressors (39, 146).

Efforts to investigate the relationship of human diseases and epigenetics - defined as changes in gene expression that might be inherited over generations of cells but are not due to an alteration of the underlying nucleotide sequence - have been growing exponentially in recent years (147). Whereas most of these studies have been undertaken in the field of cancer research, epigenetics might also provide an important concept for the pathogenetic understanding of autoimmune disorders such as RA. In RASF, epigenetic alterations have been investigated on the level of histone acetylation (99, 106, 148) and DNA methylation (95). In contrast, histone methylation has not been studied yet in RASF.

Depending on the methylated position, the methylation of histones can be associated with active or inactive chromatin regions. The trimethyl mark on histone 3 lysine 27 (H3K27me3) is generally connected with transcriptionally silenced genes. It is generated by the histone methyltransferase Enhancer of Zeste Homologue (EZH)2, the catalytic subunit of the polycomb repressor complex (PRC)2 that adds up to three methyl groups to H3K27 of genes designated for silencing (149, 150). Important targets of the PRCs include developmental genes, e.g. the transcription factors of the homeobox (*HOX*) gene family, or elements of signal transduction pathways such as the wingless-type MMTV integration site (Wnt) signalling (86, 87), which plays an important role in joint development and has been implicated in the pathogenesis of inflammatory arthropathies (57).

To address the question whether RASF show epigenetic changes on the level of histone methylation and whether these changes might contribute to their intrinsic activation, we studied the role of EZH2 in RASF.

3. Methods

A detailed Methods section is provided in the supplementary information at the end of this chapter.

Patient samples/Cell culture

Synovial tissue samples were obtained during joint surgery from RA (n=39) and osteoarthritis (OA, n=22) patients. Fibroblast cultures were maintained in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% fetal calf serum (FCS). For stimulation experiments, cells were serum-starved and stimulated with 10ng/ml tumour necrosis factor alpha (TNFα; R&D Systems, Abingdon, UK) in presence or absence of kinase inhibitors.

Quantitative real-time PCR (qPCR)

Messenger RNA expression was quantified by TaqMan or SYBR green real-time PCR on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland; primer sequences: see supplementary table S1, available online only). Data were analysed by the comparative Ct method with 18S rRNA employed as endogenous control.

SDS-PAGE and Western blot

For Western blot mouse-anti-EZH2 (Cell Signalling Technology), mouse anti-H3K27me3, rabbit anti-Histone 3, rabbit anti-secreted frizzled-related protein 1 (sFRP-1) (all from Abcam), and mouse anti-α-Tubulin (Sigma-Aldrich, Buchs, Switzerland and Abcam, Cambridge, UK) antibodies were used. Horseradish peroxidase-labelled species specific secondary antibodies (Jackson ImmunoResearch, Newmarket, Suffolk, UK) and enhanced chemiluminescence (GE Healthcare, Glattbrugg, Switzerland) were utilised for detection, and protein expression was quantified with Alpha Imager software.

Immunohistochemistry

To show expression of EZH2 *in vivo*, formalin-fixed paraffin embedded tissue sections were stained with mouse anti-EZH2 (BD Biosciences, Allschwil, Switzerland) and mouse anti-vimentin or mouse anti-CD68 (Dako, Baar, Switzerland) antibodies to demonstrate localization to fibroblasts and macrophages.

Plasmid construction

The coding sequence of EZH2 was cloned into the pcDNA3.1(+) vector (Invitrogen, Basel, Switzerland). A part of the EZH2 promoter (-1095 to +48, as described by Bracken *et al.* (151)) and the GAPDH promoter (-1087 to -24) were cloned into pGL3basic and pRL (Promega, Dübendorf, Switzerland).

Reporter gene assay

Using Nucleofector technology (Amaxa, Cologne, Germany) RASF were transfected with 0.5µg pRL_GAPDH and 2µg pGL3basic_EZH2prom (wildtype or mutated), serum-starved and stimulated with TNFα (10ng/ml) for 24h. Firefly luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) and normalized to the activity of *Renilla* luciferase.

Overexpression and gene silencing

RASF and OASF were transfected (Amaxa Nucleofection) with 1.5µg pcDNA_EZH2 or 10pmol siRNA (Qiagen, Hombrechtikon, Switzerland). Empty vector or AllStars Negative Control siRNA (Qiagen), respectively, served as controls. After 72h or 96h cells were harvested and gene overexpression or knockdown was analysed by qPCR and Western blot.

sFRP-1 enzyme-linked immunosorbent assay (ELISA)

To measure sFRP-1 in cell culture supernatants a sandwich ELISA was developed utilizing goat anti-sFRP-1 (R&D Systems) as capture antibody, rabbit anti-sFRP-1 (Abcam) as detection antibody and horseradish peroxidase-labelled goat anti-rabbit-IgG antibody (Jackson) together with 3,3',5,5'-Tetramethylbenzidin for colour development.

Chromatin immunoprecipation assay

SF were fixed with 1% formaldehyde and lysed. Chromatin was sheared by sonication and pre-cleared with normal rabbit serum (Jackson) and Protein A beads (Upstate/Millipore, Zug, Switzerland). For ChIP, 1 to 2 µg of antibody (anti-Histone 3, anti-H3K4me3 (both from Abcam), anti-H3K27me3 (Cell Signaling Technology, Danvers, MA, USA) or normal rabbit IgG (Santa Cruz, Heidelberg, Germany)) was added. Chromatin was precipitated with Protein A beads, washed, eluted, reverse-crosslinked, digested with Proteinase K and analysed by qPCR.

Statistics

For statistical analysis GraphPad Prism 5.0 software was used. Values are presented as mean±SD. A p value less than 0.05 was considered significant.

4. Results

EZH2 is constitutively overexpressed in RASF.

The histone methyltransferase EZH2 is associated with the epigenetic silencing of genes and has been found to be overexpressed in various cancers (84). Since RASF share many pathogenetic features with malignant cells, including alterations in the regulation of the cell cycle, adhesive properties, invasion to and consecutive destruction of surrounding tissues (40), we investigated the expression of EZH2 both in SF and in tissue sections from RA patients.

In vitro, we found a constitutively higher expression of EZH2 mRNA in RASF as compared to OASF (Figure 1A). These findings were confirmed on the protein level, showing an increased expression of EZH2 in RASF as analysed by Western blot (Figure 1B). Furthermore, by using synovial tissue specimen of RA and OA patients, we found that EZH2 is expressed *in vivo* as well, both in fibroblasts (stained for the mesenchymal marker vimentin, Figure 1C) and macrophages

(CD68-positive cells, Figure 1D). These data indicate that EZH2 might play a role in the epigenetic regulation of genes in RA.

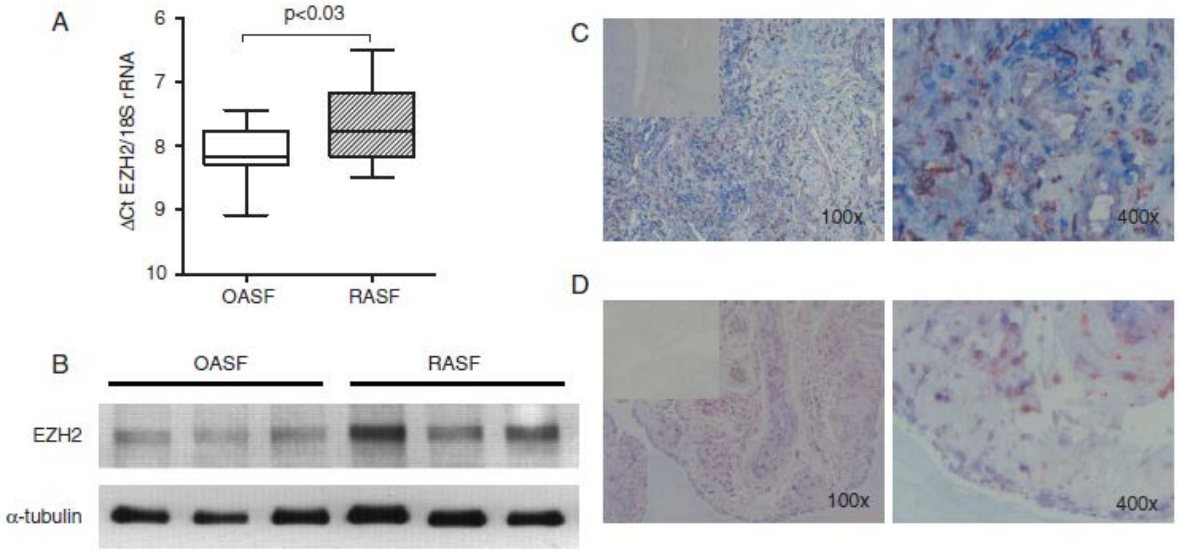

Figure 1 EZH2 expression in RASF and synovial tissue

A. EZH2 mRNA (relative to 18S rRNA) was increased by 1.5-fold in RASF (ΔCt: 7.61±0.63, n=15) as compared to OASF (ΔCt: 8.16±0.5, n=12) with higher ΔCt values representing lower expression levels (152). B. EZH2 protein was increased by 1.5-fold in RASF (n=14) as compared to OASF (n=10) (ratio EZH2/α-tubulin RASF: 0.47±0.15, OASF: 0.32±0.1, $p<0.05$). C and D. *In vivo*, EZH2 (blue) is expressed both by fibroblasts (C, Vimentin$^+$ cells, red, n=2 OA, n=2 RA) as well as by macrophages (D, CD68$^+$ cells, red, n=2 OA, n=4 RA). As a smaller inset the respective IgG control is depicted in 100x magnification.

Stimulation with TNFα induces EZH2.

TNFα is a major inflammatory mediator in the pathogenesis of RA and inflammation has already been associated with epigenetic changes (153, 154). We thus addressed whether the addition of TNFα might modulate the expression levels of EZH2 in SF. As shown in Figure 2, stimulation of cells with TNFα increased the expression of EZH2 mRNA both in RASF and in OASF with the strongest effect observed after 48h (Figure 2A). Similar results were obtained by Western blot analysis for EZH2 protein (Figure 2B). As the catalytic subunit within the PRC2, EZH2 exerts its functional activity only in connection with the other PRC2 components (155). We therefore measured the expression of other PRC2 constituents, i.e. suppressor of zeste homologue 12 (SUZ12) and embryonic ectoderm development (EED) (online Supplementary Figure S1).

The upregulation of EZH2 in SF does not correlate with global H3K27me3.

Due to the primordial function of EZH2 as a distinct methyltransferase that targets lysine 27 on histone 3, its upregulation might lead to a concomitant increase in the methylation of its substrate. By addressing the global state of H3K27 trimethylation in RASF compared to OASF by Western blot, however, no differences in H3K27me3 could be detected between patient groups or after stimulation with TNFα (Figures 2C-D).

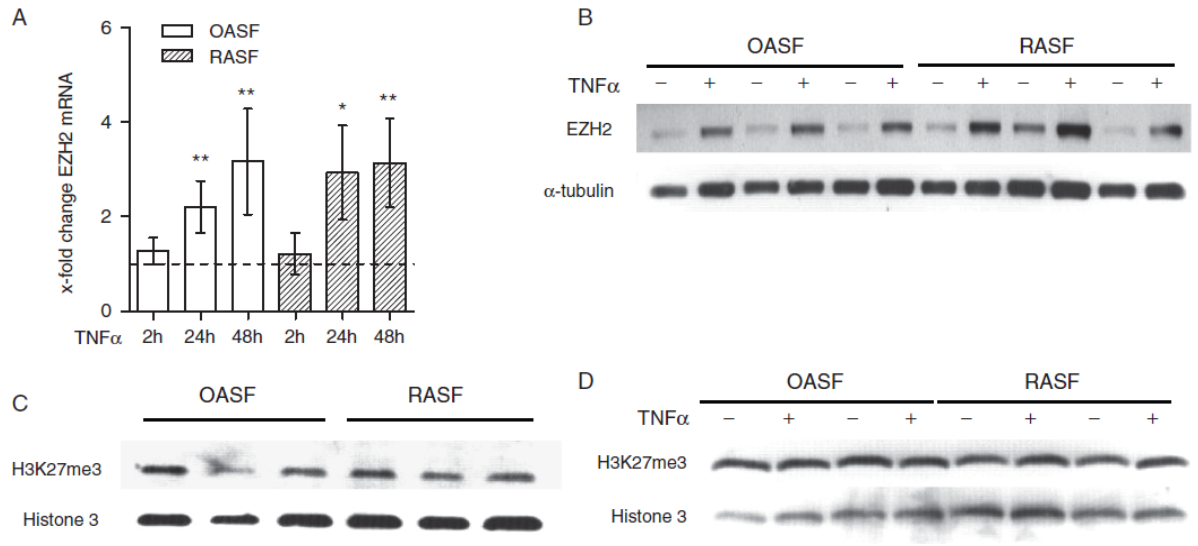

Figure 2 Effect of TNFα on EZH2 and H3K27me3

A. EZH2 mRNA was significantly induced by TNFα in OASF (2.21±0.55-fold and 3.13±0.94-fold, n=6) and RASF (2.93±1 and 3.17±1.12-fold, n=7) after 24h and 48h, respectively. The dashed line represents the unstimulated control for each time point. B. EZH2 protein was induced by TNFα in OASF (2.2±0.8-fold) and RASF (2.1±1-fold) after 48h ($p \leq 0.0001$, n=14 and 15, respectively). C. Constitutive global H3K27me3 is not different in OASF and RASF (n=3 each). D. Global H3K27me3 in TNFα stimulated OASF and RASF (n=4 each) after 48h is not changed. * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

TNFα induced upregulation of EZH2 is mediated through JNK and IKK2-dependent pathways.

In a next step we elucidated the intracellular mechanisms involved in the TNFα induced upregulation of EZH2 by performing kinase inhibition experiments for the major downstream signalling cascades of TNFα, i.e. MAP kinases and NF-κB-pathways. The inhibition of IKK-2 (inhibitor of nuclear factor κB kinase subunit beta) completely abrogated the induction of EZH2 mRNA in RASF and OASF (Figure 3A), whereas the inhibition of Jun kinase (JNK) resulted in a partial reduction of EZH2 induction (Figure 3B). Inhibition of the p38 MAP kinase did not show any effects on EZH2 expression (Figure 3C).

E2F acts as direct upstream transcriptional activator of EZH2.

It has already been shown that the transcription factor E2F is a direct upstream regulator of EZH2 in response to serum stimulation (151). Moreover, it has been reported that its direct inhibitor pRb is constitutively inactivated in RASF (156). In order to investigate whether the TNFα induced expression of EZH2 might be due to the activation of E2F, we performed reporter gene assays with luciferase vectors containing the EZH2 promoter with the wildtype or a mutated binding site for E2F (Figure 3D). TNFα stimulation resulted in a higher activity of the EZH2 promoter as compared to unstimulated control. This effect was partially inhibited by mutation of the E2F binding site, indicating a direct interaction between E2F and the EZH2 promoter.

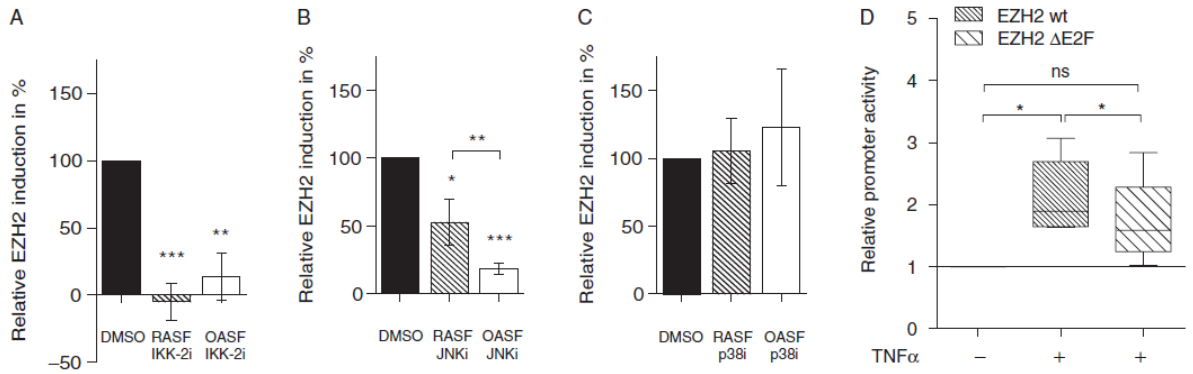

Figure 3 Regulation of TNFα induced EZH2 expression

A. Induction of EZH2 mRNA was abrogated by pretreating SF with the NF-κB pathway inhibitor sc-514 (IKK2i) (n=4 each). B. Induction of EZH2 mRNA was reduced by pretreating SF with the JNK inhibitor II (JNKi) (by 47±17% in RASF and 81±4% in OASF, n=4 each). C. Induction of EZH2 mRNA was not affected by pretreating SF with the p38 inhibitor SB203580 (p38i) (n=4 each). D. In reporter gene assays, the relative activity of the EZH2 promoter (firefly luciferase normalized to *Renilla* luciferase) in RASF (n=5) was increased after 24h TNFα stimulation by 2.1±0.6-fold. Mutation of the E2F binding site reduced the EZH2 promoter activation by TNFα to 1.7±0.7-fold. * p<0.05, ** p<0.005, *** p<0.001.

sFRP-1 is targeted by EZH2 in SF.

In the rheumatoid synovium the expression of agonists and antagonists of Wnt signalling was found to be dysregulated, among them the Wnt inhibitor sFRP-1 (157). Since elements of the Wnt pathway comprise known targets of EZH2 (86, 87), we investigated whether EZH2 might be involved in the silencing of this specific target gene in RASF. EZH2 overexpression by transfection reduced the levels of SFRP1 mRNA and protein in SF (Figures 4A-B). Conversely, transfection of RASF with siRNA targeting EZH2 resulted in a significant increase of SFRP1 mRNA at 72h (Figure 4C).

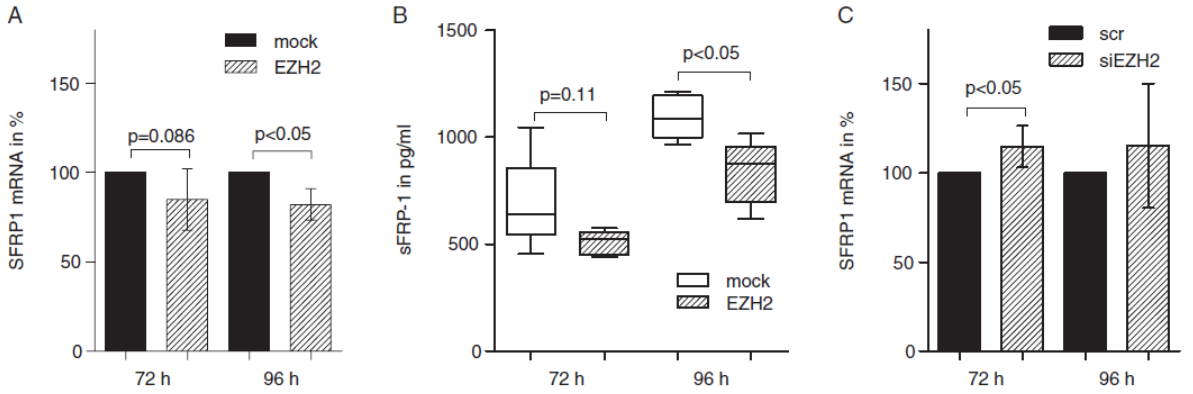

Figure 4 Overexpression of EZH2 in RASF reduces sFRP-1 expression

A. Overexpression of EZH2 decreased SFRP1 mRNA by 15±17% (72h) and 18±9% (96h). B. sFRP-1 protein in cell culture supernatants was reduced from 689±216 pg/ml to 507±56 pg/ml (minus 21.6±22.8%) after 72h and from 1095±101 pg/ml to 836±150 pg/ml (minus 22.9±17.7%) after 96h of EZH2 transfection, as assessed by ELSIA. C. Knockdown of EZH2 resulted in a significant upregulation of SFRP1 mRNA by 15±11.7% at 72h.

sFRP-1 is differentially expressed in RASF and OASF and is strongly repressed by TNFα.

To test the hypothesis that SFRP1 might be epigenetically silenced in RASF, its differential expression between RASF and OASF was assessed. As shown in Figure 5A the expression of SFRP1 mRNA was significantly reduced in RASF compared to OASF. Figures 5B and C emphasize that this was also true for the expression of sFRP-1 protein with RASF reaching only half the sFRP-1 expression observed in OASF.

Since EZH2 is upregulated by TNFα, and sFRP-1 appeared to be a target of EZH2-mediated gene silencing, we asked whether TNFα also has an impact on the expression of sFRP-1. As shown in Figures 5D and E stimulation of SF with TNFα strongly decreased the expression of SFRP1 mRNA and protein after 48h. Yet, a direct link between the TNFα induced upregulation of EZH2 and the downregulation of SFRP1 remains presumptive at this moment.

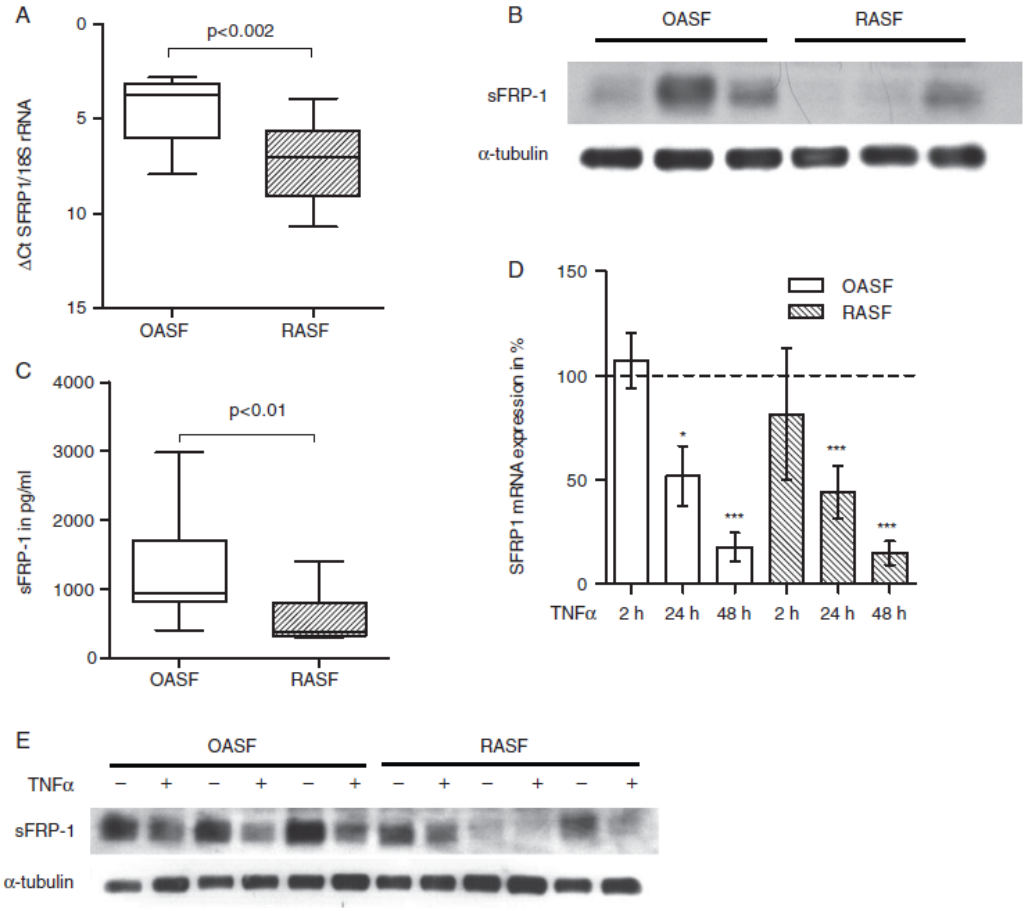

Figure 5 Constitutive and TNFα regulated expression of sFRP-1 in RASF and OASF

A. SFRP1 mRNA (relative to 18S rRNA) was reduced by 86% in RASF (ΔCt: 7.1±2, n=15) as compared to OASF (ΔCt: 4.5±1.7, n=12). B. Western blot analysis showed strongly reduced levels of sFRP-1 protein in RASF (n=10) compared to OASF (n=7) (ratio sFRP-1/α-tubulin RASF: 0.31±0.18, OASF: 0.63±0.31, $p<0.05$). C. sFRP-1 levels in supernatants of RASF (566±354 pg/ml, n=10) were reduced by 56% compared to OASF (1285±845 pg/ml, n=7). D. SFRP1 mRNA levels were reduced by TNFα in RASF (by 85±6%, n=6) and OASF (by 82±7%, n=7) with the strongest effect after 48h. The dashed line represents the respective unstimulated control for each time point E. sFRP-1 protein expression was decreased after 48h of TNFα stimulation in RASF (by 36±19%) and OASF (by 45±7%) (n=6 each, $p\leq0.005$). * $p<0.05$, ** $p<0.005$, *** $p<0.001$

Activating and silencing chromatin marks of the SFPR1 gene promoter are different between RASF and OASF.

To prove that SFRP1 is subject to EZH2 mediated silencing we performed chromatin immunoprecipitation (ChIP) analysis. Chromatin precipitated with antibodies against histone 3, H3K4me3 and H3K27me3 was analysed for enrichment in three regions of the SFRP1 promoter (upstream, downstream and at the transcription start site (TSS), Figure 6A). In general, the differences in H3K4me3 and H3K27me3 occupation of the SFRP1 promoter between RASF and OASF were strongest in region 3. Whereas the activating methylation of H3K4 was reduced in RASF, the repressive H3K27 trimethylation was increased (Figure 6B). The trends observed in promoter regions 1 and 2 were similar but weaker than in region 3.

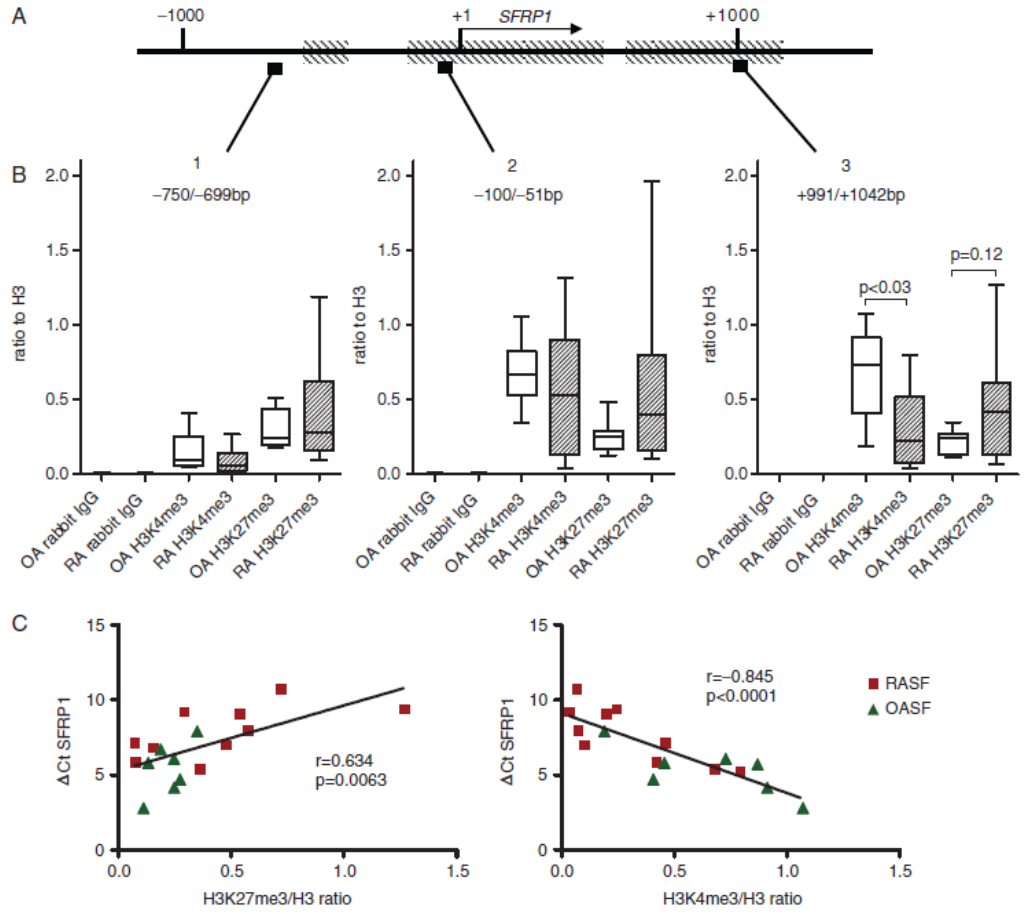

Figure 6 ChIP analysis of the SFRP1 promoter in RASF and OASF

A. Schematic representation of the SFRP1 promoter. Shaded areas represent CpG islands as calculated by MethPrimer (www.urogene.org/methprimer). "+1" indicates the transcription start site (TSS). Black boxes represent the three different amplicons for ChIP-qPCR (1: -750/-699 bp, 2: -100/-51 bp, 3: +991/+1024 bp relative to TSS) B. qPCR analysis of chromatin from RASF (n=10) and OASF (n=7) ChIPed with rabbit IgG, anti-Histone 3, anti-H3K4me3 and anti-H3K27me3. To compare different patient samples, the ratio between the levels of the methylation mark and total histone 3 at promoter regions was calculated. RASF showed a decreased trimethylation of H3K4 (ratio to H3: 0.31±0.27, OASF: 0.66±0.32) and increased H3K27me3 (ratio to H3: 0.45±0.36, OASF: 0.22±0.08). C. ΔCt of SFRP1 mRNA in RASF (■) and OASF (▲) correlated negatively with the H3K4me3/H3 ratio (right) and positively with the H3K27me3/H3 ratio (left) in region 3 of the SFRP1 promoter.

Since we observed a strong variability of H3K4me3 and H3K27me3 at the SFRP1 promoter between cells from different patients, we analysed whether this might correlate with the expression of SFRP1. As shown in Figure 6C, a strong positive correlation between H3K27me3 and the ΔCt value of SFRP1 was found (Pearson's r=0.634). On the other hand, a high H3K4me3/H3 ratio correlated with a low SFRP1 ΔCt value and thus higher mRNA expression levels (r=-0.845). These data indicate that SFRP1 expression in RASF is reduced due to decreased H3K4me3 and increased H3K27 methylation in its promoter.

We also analysed other Wnt inhibitors, including Dickkopf-1 (DKK1) and SFRP2-5, but could only detect significant expression differences for SFRP2 (online Supplementary Figure S2). Although there was a general trend towards a correlation between H3K4me3 and H3K27me3 occupation of the promoters and Wnt inhibitor expression levels, there was not such a distinct pattern of expression correlating with both histone methylation marks as for SFRP1. These data suggest that the differential regulation by H3K4/H3K27 methylation in OASF and RASF is unique to SFRP1.

5. Discussion

In this study we focused on the expression and function of the histone methyltransferase EZH2 in synovial fibroblasts of RA patients and found that a) EZH2 is overexpressed in RASF as compared to OASF; b) expression of EZH2 is induced by TNFα involving NF-κB and MAP-kinase pathways; and c) SFRP1 is a direct target gene of EZH2, whose expression pattern correlates with the occupation of activating and inactivating histone methylation marks in its promoter region.

Whereas the status of histone methylation and its respective mediators has not been assessed in RA so far, EZH2 has been found overexpressed in different cancers and, in malignancies involving the prostate and breast, it has been proposed as a marker of disease progression and metastatic potential (158, 159). Likewise, the behaviour of cells within the rheumatoid synovium has been compared to the growth of an invasive tumour from which synovial fibroblasts actively invade and destroy the articular cartilage (1, 42, 160). Consistent with the findings from cancer tissues, we showed in the present study that the expression levels of EZH2 are increased in RASF and, moreover, that these levels can be further enhanced by TNFα stimulation. We could link this effect to the intracellular activation of NF-κB, JNK and E2F-Rb pathways, all of which represent major signalling cascades involved in the activation of SF.

Despite the induction of EZH2 expression, we could not detect changes of global H3K27me3 in response to TNFα. One thus could speculate that EZH2 is specifically targeted to certain chromatin areas so that its activity is eventually not reflected in global changes of H3K27me3. Such specific targeting of local chromatin areas has already been suggested by others. De Santa *et al*. could demonstrate that in macrophages the LPS-induced expression of Jmjd3, i.e. the demethylase for H3K27me3, did not cause alterations in global methylation of H3K27 (153). In a next step we thus

focused on potential target genes that might show local methylation changes in their promoters and identified SFRP1 as a direct target of EZH2. Decreased expression of sFRP-1 within the rheumatoid synovium has already been described by Imai *et al.*(157); here, we identified the distinct epigenetic mechanisms of this downregulation and unravelled the signalling pathways in RASF *in vitro*.

sFRP-1 inhibits Wnt signalling by directly binding to Wnts and is thus believed to antagonize both the canonical and non-canonical pathways (161). Inactivation of SFRP1 by DNA methylation has been described among the early changes occurring in the development of colorectal cancer (162). In RA, canonical Wnt signalling has been shown to participate in the activation of RASF through the regulation of cytokines, matrix metalloproteinase (MMP)3 and production of extracellular matrix molecules (163, 164). Furthermore, the RA synovium has been found to contain higher levels of Wnt1, Wnt5A, and Wnt10B than OA or normal synovium (157, 165). It is thus highly suggestive that, by producing considerably less amounts of sFRP-1, the RASF is turned even more responsive to Wnt proteins in the synovial tissue. Inhibition of Wnt agonists or upregulation of Wnt antagonists, e.g. by interfering with epigenetic mechanisms, therefore appears to be favourable in the pathogenesis of RA regarding the attenuation of the invasive and destructive potential of RASF. On the other hand, bone erosions can be inhibited by blocking the Wnt inhibitor DKK1 which was found to enhance osteoclastogenesis and repress bone formation by osteoblasts (56, 166). The question thus arises whether the influence of Wnt signalling on joint destruction is cell type dependent or defined by the different modes of action of DKK1 and SFRPs.

Our data indicate that the epigenetic landscape of the SFRP1 promoter in RASF is changed towards a transcription-inhibiting state. Overexpression of the H3K27-methyltransferase EZH2 led to reduced levels of SFRP1 and, conversely, silencing of EZH2 to increased SFRP1 levels. By comparing RASF with OASF, we could observe increased H3K27me3 with a concomitant decrease of H3K4me3. H3K4me3 and H3K27me3 are known to co-localise in bivalent domains which, depending on the type of cells, cause genes to tend towards a rather repressed or activated state (76, 77). Ke *et al.* showed that genes carrying H3K4me3 are more expressed than those carrying both H3K4me3 and H3K27me3, which in turn are more highly expressed than those carrying only H3K27me3 (77). Indeed, we have demonstrated that the SFRP1 promoter in SF contains such a bivalent domain and that the expression of SFRP1 correlates with both methyl marks.

Our experiments suggest the following model of SFRP1 silencing in SF in the chronically inflamed RA joint. Continuous exposure to TNFα and other inflammatory mediators (see online Supplementary Figure S3) might lead to alterations in epigenetic programmes, e.g. through the induction of EZH2 expression. Trimethylation of lysine 27 of histone 3 and the loss of H3K4 trimethylation within the promoter regions might implement the TNFα induced silencing of SFRP1 so that it could persist even outside the inflammatory environment. Whereas only a weak correlation between the EZH2 expression and SFRP1 expression was found, the levels of SFRP1 mRNA and methylation status of its promoter could be strongly correlated. In cell culture, RASF escape from the

ongoing exposure to inflammatory cytokines thus probably leading to a relative normalization of the EZH2 levels. This could explain the proportionally moderate increase of basal EZH2 expression in RASF when compared to OASF as observed in the present study. These basal expression levels of EZH2, however, might already be sufficient to maintain the epigenetic memory of the cells by copying the methyl mark on the SFRP1 gene during replication. Whether the addition of TNF antagonists in patients might have a similar effect and, over time, even reverse the epigenetic phenotype is speculative. Since our data suggest an analogous role of EZH2 in RASF and in cancer cells, it might be of interest for further studies to investigate EZH2 as potential marker for disease activity, in particular for patients with ongoing joint destruction despite treatment with TNF antagonists.

In summary, we addressed here for the first time histone methylation in RASF and found an overexpression of the histone methyltransferase EZH2 thus leading to alterations in the Wnt signalling pathway by downregulation of the tumour suppressor gene SFRP1.

Acknowledgements

We thank Dr. Britta Maurer and Emmanuel Karouzakis for valuable discussions. This work was supported by the Swiss National Science Foundation (SNSF-32000-116842), Schwyzer Foundation, Autocure FP6, Masterswitch FP7 and IAR Epalinges programmes.

6. Supplementary Information

Supplementary Methods

Patient samples/ Cell culture

Synovial tissue samples were obtained during joint surgery from RA and osteoarthritis (OA) patients after they had given written informed consent for further use of their tissue for research purposes. Work with patient materials was approved by the local ethics committee. For fibroblast cultures, tissue specimen were digested with collagenase and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Fibroblasts from passages 4 to 9 were used.

Stimulation experiments/ Inhibitor studies

SF were starved for 16-24h in DMEM containing 0.5% FCS and stimulated with 10ng/ml TNFα (R&D Systems). For kinase inhibition studies, SF were pre-incubated with sc-514 (50μM, IKK-2 inhibitor), JNK Inhibitor II (20μM), p38 inhibitor SB203580 (10μM) (all from Sigma Aldrich) or DMSO alone for 1h before adding TNFα.

SDS-PAGE and Western blot

Whole cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose or PVDF membranes. Membranes were blocked with 5% milk or 2% horse serum in TBS-T and incubated with the following antibodies: mouse-anti-EZH2 (Cell Signaling Technology), mouse-anti-H3K27me3, rabbit-anti-Histone 3, rabbit-anti-SUZ12, rabbit-anti-sFRP-1 (all from Abcam), sheep-anti-EED (R&D Systems), mouse-anti-α-Tubulin (Sigma-Aldrich and Abcam). Horseradish peroxidase (HRP)-labelled species specific secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (GE Healthcare) were utilised for detection, and protein expression was quantified with Alpha Imager software.

Immunohistochemistry

Formalin-fixed paraffin embedded tissue sections were deparaffinised and underwent antigen retrieval with 1mg/ml Trypsin for 25 min at 37°C (only for CD68 staining) and in 10mM citrate buffer (pH 6.0) for 30 min at 90°C. Unspecific binding was blocked by 5% goat serum in PBS (+1% BSA) before the sections were incubated with mouse-anti-EZH2 (BD Biosciences) or mouse IgG (Dako) (10µg/ml) over night at 4°C. Biotinylated secondary goat-anti-mouse antibody (Jackson ImmunoResearch), Vectastain ABC Kit for alkaline phosphatase and Vector Blue (Vector Laboratories) were used for detection. Subsequently, endogenous peroxidase was blocked with 3% H_2O_2 for 10 min before incubating with 5% goat serum in PBS (+1% BSA) for 1h. To show the expression of EZH2 in cells of mesenchymal origin (fibroblasts) or in macrophages, mouse-anti-vimentin or mouse-anti-CD68 (2µg/ml, Dako) antibodies were applied over night at 4°C and detected using HRP-labelled goat-anti-mouse antibody (for vimentin) or biotinylated goat-anti-mouse antibody and Vectastain Elite ABC Kit for HRP (for CD68) together with aminoethylcarbazole as chromogen.

Plasmid construction

The coding sequence of EZH2 was amplified from human cDNA and inserted into the pcDNA3.1(+) vector (Invitrogen) via the *BamH*I and *Xho*I restriction sites. A part of the EZH2 promoter (-1095 to +48, as described by Bracken *et al*. (151)) and the GAPDH promoter (-1087 to -24) were amplified from human genomic DNA and cloned into pGL3basic and pRL (Promega), respectively, via the *Bgl*II and *Hind*III sites. The E2F binding site (+33 to +40) in the EZH2 promoter was mutated by site-directed mutagenesis in four positions.

Transfection

RASF and OASF were transfected with the Basic Nucleofector Kit for Primary Mammalian Fibroblasts (Amaxa) according to the manufacturer's instructions. The medium was replaced 24h after transfection to remove floating cells.

Reporter gene assay

RASF were transfected with 0.5µg pRL_GAPDH and 2µg pGL3basic_EZH2prom or pGL3basic_EZH2prom_ΔE2F. After 24h in complete medium, cells were starved for 24h (DMEM + 0.5% FCS) and stimulated with TNFα (10ng/ml) for another 24h. Firefly luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) and normalized to the activity of Renilla luciferase.

sFRP-1 enzyme-linked immunosorbent assay (ELISA)

To measure sFRP-1 in cell culture supernatants microwell plates were coated with goat-anti-sFRP-1 antibody (0.5mg/ml, R&D Systems) in sodium carbonate buffer (pH 9.5) over night at 4°C (as in (167)). PBS with 0.05% Tween-20 was used for the washing between incubation steps. After coating, wells were blocked with 300µl reagent diluent (2% non-fat dry milk in PBS) for 1h at room temperature. Recombinant sFRP-1 (R&D Systems) was used to generate a standard curve (range: 0-10000 pg/ml) and incubated together with the undiluted cell culture supernatants for 2h at room temperature. For detection, rabbit-anti-sFRP-1 antibody (1:1000, Abcam) was added for 2h. HRP-labelled goat-anti-rabbit-IgG antibody (1:5000, Jackson) was incubated for 1h and detection was performed with 3,3',5,5'-Tetramethylbenzidin. Colour development was stopped with 2N H_2SO_4 and absorbance was measured at 450 nm (with wavelength correction at 560 nm).

Chromatin immunoprecipation assay

Three million SF were fixed with formaldehyde solution for 10 min (see online Supplementary Table S2 for details of buffer compositions). Fixation was stopped by adding 200mM glycine. After washing the cells with PBS three times, lysis buffer was added. Chromatin was sheared by sonication to a size of 400-800 bp (Bandelin Sonopuls HD2070, 6 cycles of 10s sonication at 50% power and 1 min pause on ice) and 10% of the sample was removed as input control. Sonicated chromatin was diluted 1:10 and pre-cleared for 4h by adding 10µl/ml normal rabbit serum (Jackson) and 20µl/ml Protein A beads (Upstate, 50% slurry in TE blocked with 1mg/ml BSA and 0.4mg/ml herring sperm DNA). Pre-cleared chromatin was parcelled to aliquots, 1 to 2 µg of antibody (anti-Histone 3, anti-H3K4me3 (both from Abcam), anti-H3K27me3 (Cell Signaling Technology) or normal rabbit IgG (Santa Cruz) was added and rotated over night at 4°C. Chromatin was precipitated with 40µl/ml Protein A beads for 4h, washed with wash buffers I to III and finally two times TE buffer. Precipitated chromatin was eluted by incubating twice with elution buffer for 20 min, reverse cross-linked and digested with Proteinase K over night at 65°C and purified with the PCR purification kit (Qiagen). Samples were analysed by qPCR and normalized to the 10% input sample. Occupation of the GAPDH and MYOD promoters was measured as positive controls for H3K4me3 and H3K27me3, respectively.

Statistics

For statistical analysis GraphPad Prism 5.0 software was used. Values are presented as mean ± standard deviation. Data were analysed using Student's t-test for parametric samples and the Mann-Whitney test or Wilcoxon signed rank test for nonparametric samples, as well as Pearson's correlation. A p-value < 0.05 was considered significant. Detailed information about which statistical test was used for individual experiments are provided in Supplementary Table S3.

Supplementary Figures

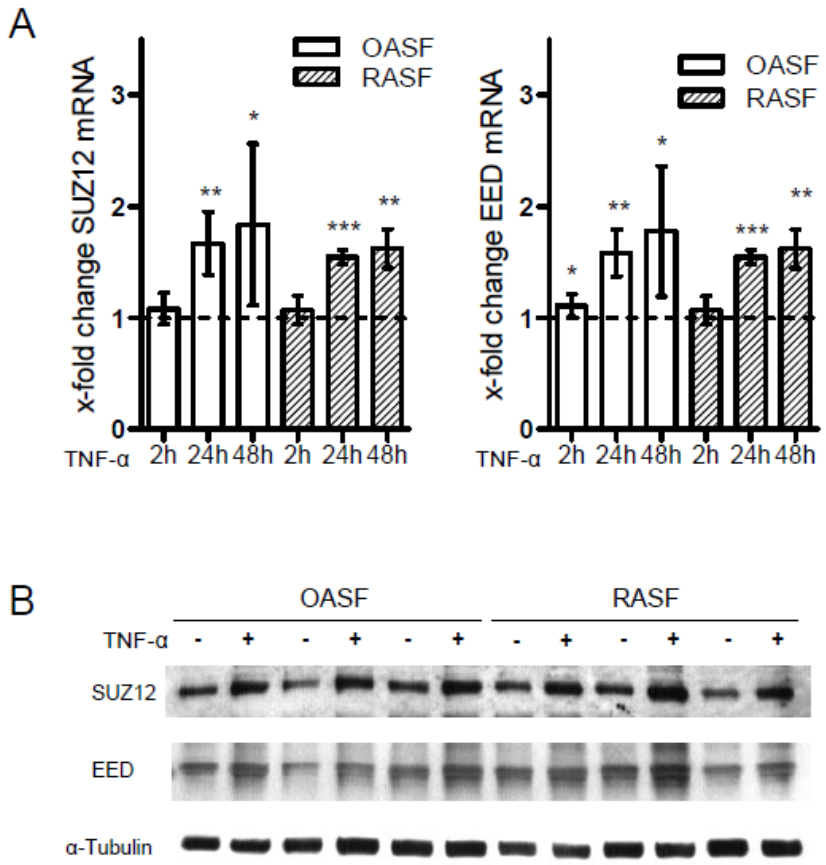

Figure S1 Effect of TNFα on the PRC2 components EED and SUZ12

A. SUZ12 and EED mRNAs were significantly induced by TNFα in OASF (n=6) and RASF (n=7) after 24h and 48h. B. EED (n=5 OASF, n=8 RASF) and SUZ12 protein expression after 48h TNFα stimulation. SUZ12 protein was induced in OASF (2.6±2.2-fold, p=0.001, n=11) and RASF (1.8±0.8-fold, p=0.0002, n=14).

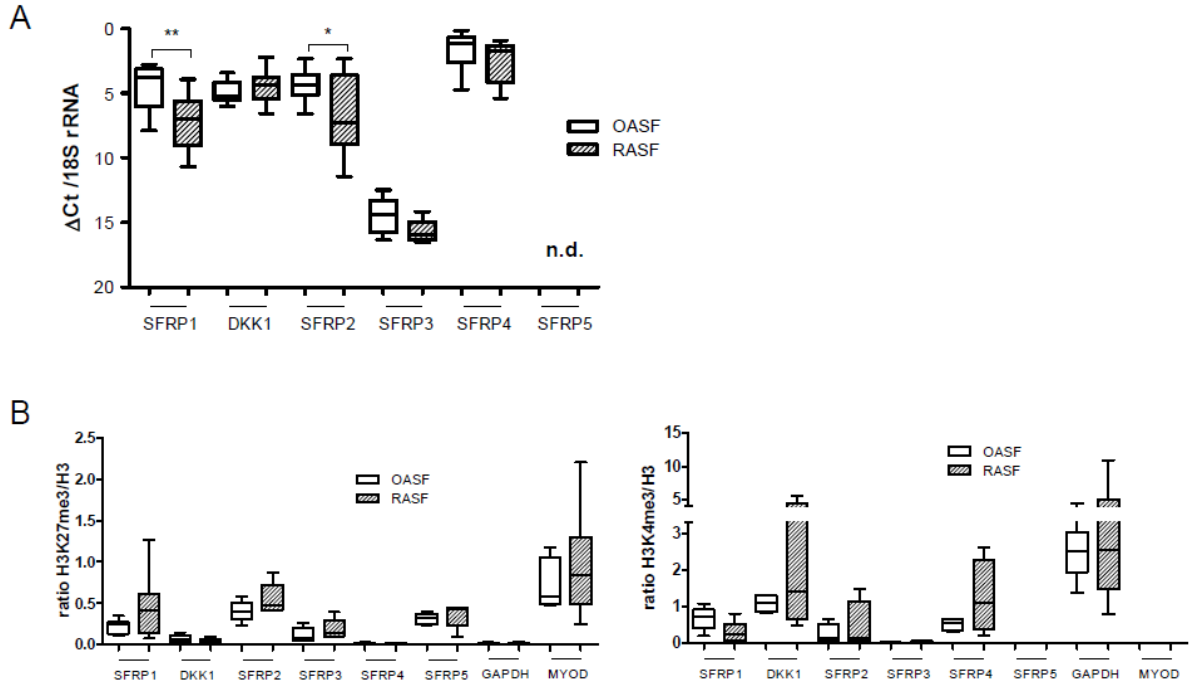

Figure S2 mRNA expression of different Wnt inhibitors and occupation of their promoters with H3K27me3 and H3K4me3

A. Similar to SFRP1, the expression of SFRP2 was significantly reduced in RASF. Also, SFRP4, which showed the highest expression of all Wnt inhibitors measured (ΔCt RASF: 2.43±1.54, OASF: 1.67±1.3), was reduced in RASF; the reduction observed, however, did not reach statistical significance. In contrast, DKK1 showed a trend towards higher expression in RASF. SFRP3 was only weakly expressed and levels of SFRP5 mRNA could not be detected (n.d.). B. The occupation of the SFRP2 promoter with H3K27me3 was comparable to that of SFRP1. The promoters of DKK1 and SFRP4 showed low levels of H3K27me3. The H3K27me3 occupation of the promoters for SFRP2, SFRP3 and SFRP5 tended to be higher in RASF than in OASF; these differences were not statistically significant. Interestingly, the activating H3K4me3 mark was absent at the SFRP3 and SFRP5 promoters in both OASF and RASF, which may – at least in part – explain the low (SFRP3) or missing (SFRP5) mRNA expression of these Wnt inhibitors. In contrast, the DKK1 and SFRP4 gene promoters contained high levels of H3K4me3, most likely accounting for their high mRNA expression levels. However, different to SFRP1, we could not correlate H3K4me3/H3 and H3K27me3/H3 ratios of DKK1 and SFRP2-5 to their respective mRNA expression levels. H3K4me3 and H3K27me3 levels at the promoters of GAPDH and MYOD are shown as positive (GAPDH for H3K4me3, MYOD for H3K27me3) and negative controls (GAPDH for H3K27me3, MYOD for H3K4me3), respectively. (RASF: n=4-15, OASF: n= 4-12)

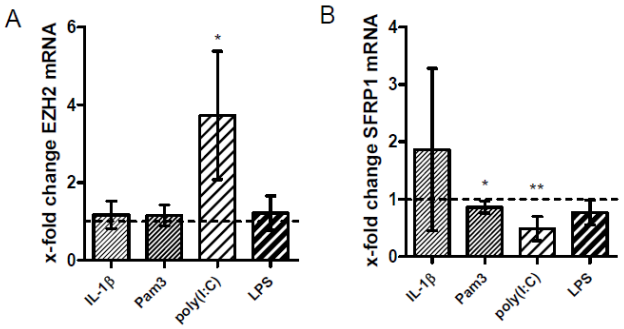

Figure S3 Effect of interleukin-1β (IL-1β) and toll-like receptor (TLR) ligands on the expression of EZH2 and SFRP1

Similar to stimulation with TNFα, treatment of RASF (n=5) with the TLR3 ligand poly(I:C) led to upregulation of EZH2 (A) and downregulation of SFRP1 (B) after 48h. Additionally, SFRP1 mRNA expression was reduced by stimulation with the TLR2 ligand Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH (Pam3). IL-1β and the TLR4 ligand lipopolysaccharide (LPS) did not have an effect on the mRNA levels of EZH2 or SFRP1. The dashed line represents the unstimulated control. * $p<0.05$, ** $p<0.005$.

Supplementary Tables

Table S1 Primers for qPCR

gene expression analysis	
18S rRNA	assay from Applied Biosystems (Cat. No. 4310893E)
DKK1 for	5’ - CCA GAC CAT TGA CAA CTA CCA G – 3’
DKK1 rev	5’ - TGC CGC ACT CCT CGT CCT C – 3’
EZH2 for	5’ – AGT GTT ACC AGC ATT TGG AGG G – 3’
EZH2 rev	5’ – CGG TGA GAG CAG CAG CAA AC – 3’
EED for	5’ - GCA ATC CAG ACC TCT CTG GAG - 3’
EED rev	5’ - CCA CTT TCT ATA CTG ACA GCG TC - 3’
SFRP1 for	5' - TGA GGC CAT CAT TGA ACA TC - 3'
SFRP1 rev	5' - TCA TCC TCA GTG CAA ACT CG - 3'
SFRP2 for	5’ - AGC GAC CAC CTC CTG CCA G – 3’
SFRP2 rev	5’ - CAG GCT TCA CAT ACC TTT GGA G – 3’
SFRP3 for	5’ - CTA GTA ACG GAA ACT GTA GAG GG – 3’
SFRP3 rev	5’ - CTT ACA TTT ACA GCG TTC ACT GC – 3’
SFRP4 for	5' - GCC TTT CCT GTA CCA TCA TGT C - 3'
SFRP4 rev	5' - CCG GAG GAT GTT AAG TGG ATA G - 3'
SFRP5 for	5’ - TGC TCC AGT GAC TTT GTG GTC – 3’
SFRP5 rev	5’ - CCC ATT CTC TAT CTT GAT CTC C – 3’
SUZ12 for	5’ - GGG AGA CTA TTC TTG ATG GGA AG - 3’
SUZ12 rev	5’ - GAG AAA ATG TTT CGA ATG GAG GC - 3’
ChIP analysis	
DKK1 TSS for	5’ - GTA TAA AGG CAG CCG CGG TG – 3’
DKK1 TSS rev	5’ - TCC CAG AGT CCT GAC TGC AG – 3’
GAPDH for	5’ - TCG ACA GTC AGC CGC ATC TTC – 3’
GAPDH rev	5’ - CTA GCC TCC CGG GTT TCT CT – 3’
MYOD for	5’ - CCG CCT GAG CAA AGT AAA TGA G – 3’
MYOD rev	5’ - TGG GCA ACC GCT GGT TTG GAT – 3’
SFRP1 up for (region 1)	5’ - CTT CCT GAA CCC CTG TTA GTT G – 3’
SFRP1 up rev (region 1)	5’ - TCT AGC TGC GGT GCA GAC AG – 3’
SFRP1 TSS for (region 2)	5’ - TGA CGG ACG TGG TAA CGA GTG – 3’
SFRP1 TSS rev (region 2)	5’ - AGC CAA TCA GCT CCC GGC G – 3’
SFRP1 down for (region 3)	5’ - CGT GAT TCC TAA GCG CGT CTG – 3’
SFRP1 down rev (region 3)	5’ - GAG GGG CGC TGA GCG ATA C – 3’
SFRP2 TSS for	5’ - CCG GTG TCC CGC TTC TCC – 3’
SFRP2 TSS rev	5’ - TCT TCG CTG GGT GCG ACT C – 3’
SFRP3 TSS for	5’ - TTG GGA AAG AGC AGC CTG GG – 3’
SFRP3 TSS rev	5’ - CTT TAC CGA GCT CCA GCC AC – 3’
SFRP4 TSS for	5’ - AGC TGC CAA GGG AGC GTT C – 3’
SFRP4 TSS rev	5’ - CCA TTG CGG GAC CCT ATT TAT C – 3’
SFRP5 TSS for	5’ - GAG GAA AAT GCC CAG GGA GG – 3’
SFRP5 TSS rev	5’ - CCT CGC CCA GCG TTT CTC C – 3’

Table S2 Buffers for ChIP

Formaldehyde Solution	**Lysis buffer ***	**Dilution Buffer***	**Wash Buffer 1***
1% formaldehyde 4.5mM HEPES pH 8.0 9mM NaCl 0.09mM EDTA 0.045mM EGTA in PBS	50mM Tris/HCl pH 8.0 1% SDS 5mM EDTA	20mM Tris/HCl pH 8.0 150mM NaCl 2mM EDTA 1% Triton X-100	20 mM Tris-HCl pH 8.0 150 mM NaCl 2 mM EDTA 1% Triton X-100 0.1% SDS
Wash Buffer 2*	**Wash Buffer 3***	**Elution Buffer**	
20 mM Tris-HCl pH 8.0 500 mM NaCl 2 mM EDTA 1% Triton X-100 0.1% SDS	10 mM Tris-HCl pH 8.0 0.25 M LiCl 1 mM EDTA 1% deoxycholate 1% NP-40	0.1M $NaHCO_3$ 1% SDS	

*supplemented with 1x Protease Inhibitor Cocktail (Roche)

Table S3 Statistical tests

test used / **Figure**		**student's t-test**	**Mann-Whitney test**	**Wilcoxon signed rank test**	**Pearson's correlation**
1	A	✓			
	B	✓			
2	A	✓			
	B	✓(OASF)		✓(RASF)	
3	A	✓			
	B	✓			
	C	✓			
	D	✓			
4	A	✓			
	B	✓			
	C	✓			
5	A	✓			
	B	✓			
	C		✓		
	D	✓			
	E	✓			
6	B	✓			
	C				✓

CHAPTER III

The TNFα-induced miR-18a activates rheumatoid arthritis synovial fibroblasts through a feedback loop in NF-κB signalling

Michelle Trenkmann, Matthias Brock, Renate E. Gay, Beat A. Michel, Steffen Gay, Lars C. Huber. *Arthritis Rheum.* in press

1. Abstract

Objective: To elucidate whether the microRNA (miRNA) cluster miR-17-92 contributes to the activated phenotype of rheumatoid arthritis synovial fibroblasts (RASF).

Methods: RASF were stimulated with tumor necrosis factor alpha (TNFα) and expression and regulation of the miR-17-92 cluster were studied using quantitative real-time PCR (qPCR) and promoter activity assays. RASF were transfected with single precursor molecules of miRNAs from miR-17-92 and the expression of matrix-degrading enzymes and cytokines was measured by qPCR and enzyme-linked immunosorbent assay. Potential miRNA targets were identified by computational prediction and validated using reporter gene assays and Western blot. The activity of NF-κB signaling was determined by reporter gene assay.

Results: We found that TNFα induces the expression of miR-17-92 in RASF in a nuclear factor kappa B (NF-κB)-dependent manner. Transfection of RASF with precursor molecules of single members of miR-17-92 revealed significantly increased expression levels of matrix-degrading enzymes, proinflammatory cytokines and chemokines in pre-miR-18a-transfected RASF. Using reporter gene assays we identified the NF-κB pathway inhibitor TNFα-induced protein 3 (TNFAIP3) as a new target of miR-18a. In consequence, pre-miR-18a-transfected RASF showed stronger activation of NF-κB signaling, both constitutively and in response to TNFα-stimulation.

Conclusion: Our data suggest that the miR-17-92-derived miR-18a contributes to cartilage destruction and chronic inflammation in the joint through a positive feedback loop in NF-κB signaling with concomitant upregulation of matrix-degrading enzymes and inflammatory mediators in RASF.

2. Introduction

The microRNA-17-92 (miR-17-92) cluster has been associated with the maturation of the immune system and the development of hematopoietic tumors and other malignancies (142, 168, 169). The polycistronic primary transcript C13orf25 encodes six distinct members of the cluster: miR-17, -18a, -19a, -19b, -20a and -92a (168, 170). Amplification of miR-17-92 has been observed in malignant diseases leading to growth and survival advantages due to the silencing of tumor suppressor genes such as the cyclin-dependent kinase inhibitor 1A (p21), phosphatase and tensin homolog (PTEN) or Bcl-2 interacting protein Bim by individual miR-17-92 members (168, 169, 171-173). miR-17-92 knockout mice, on the other hand, displayed skeletal defects during embryogenesis (174) and died directly after birth due to severe developmental defects of the lung and the heart (175), whereas a moderate overexpression of miR-17-92 in the lymphoid lineage led to lymphoproliferative disease and autoimmunity in mice (171). Interestingly, the expression of miR-17-92 is triggered by inflammatory cytokines such as interleukin (IL)-6 (143, 145).

Rheumatoid arthritis (RA) is a chronic inflammatory disease ultimately leading to the destruction of joints and bones. The etiology of RA is unknown so far; it is clear, however, that the disease is driven by infiltrating immune cells as well as resident cells of the joint, and the mutual interaction between these cells (1). In this regard, RA synovial fibroblasts (SF) have been identified as pathogenetic key players. Within the inflamed synovium, RASF are stimulated by cytokines and other molecules to activate major signaling pathways, which leads to overproduction and secretion of inflammatory mediators and matrix-degrading enzymes. Immune cells are attracted to the synovium and activated by RASF-secreted cytokines and, in turn, stimulate RASF resulting in a self-sustaining cycle of chronic joint inflammation (19). Due to the disturbed expression pattern of various proto-oncogenes and tumor suppressors the aggressive phenotype of the RASF has been compared with the behavior of a locally invading tumor (42), and, as such, these cells promote adhesion to and destruction of articular cartilage (1, 39). Similar to the pathogenesis of malignant tumors, an aberrant expression of several microRNAs (miRNAs) has recently been associated with the pathogenesis of RA (176).

miRNAs are characterized by their ability to target different mRNAs simultaneously, which might influence several signaling pathways at once. miRNAs are considered to act by fine-tuning gene expression; the effects of a single miRNA on a specific target thus might be rather weak, however, by repressing many targets at the same time a significant alteration of gene expression can occur (113, 177).

We have previously shown that IL-6 induces miR-17-92 both in pulmonary arterial endothelial cells and in human hepatocytes, in the latter ones enhancing the acute-phase response (143, 145). Thus, to further elucidate the role of miR-17-92 in inflammatory processes we studied here expression and

function of miR-17-92, in particular of miR-18a, in RASF in relationship with tumor necrosis factor alpha (TNFα), one of the major pro-inflammatory cytokines in the pathogenesis of RA (48, 54).

3. Materials and Methods

Patient samples/ Cell culture

Synovial fibroblast cultures were established by collagenolytic digestion of synovial tissue specimens obtained during joint surgery from RA patients fulfilling the 1987 American College of Rheumatology criteria for RA (16) (kindly provided by Dr. C. Kolling of the Schulthess Clinic, Zurich, Switzerland). Fibroblast cultures were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) or in Roswell Park Memorial Institute 1640 (RPMI) medium (only for leukocyte transwell migration assay). For stimulation experiments, cells were serum-starved (0.5% FCS) and stimulated with 10ng/ml TNFα (R&D Systems). To inhibit NF-κB signaling, RASF were treated with 50 μM sc-514 (Merck-Millipore) (or DMSO as control) for 1h before TNFα was added.

Quantitative real-time PCR

mRNA and miRNA expression were quantified by SYBR Green real-time polymerase chain reaction (PCR) on the ABI Prism 7900 HT Sequence Detection System. Primer sequences were: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 5'-GGGAAGCTTGTCATCAATGGA-3', GAPDH rev 5'-TCTCGCTCCTGGAAGATGGT-3', C13orf25 for 5'-TTGCTAAGTGGAAGCCAGAAG-3', C13orf25 rev 5'-CATCCACGTGGCAAAACAT-3' (as described by O'Donnell *et al.* (178)), matrix metalloproteinase (MMP) 1 for 5'-GCTAACAAATACTGGAGGTATGATG-3', MMP1 rev 5'-ATTTTGGGATAACCTGGATCCATAG-3', IL6 for 5'-CCCTGAGAAAGGAGACATGTAAC-3', IL6 rev 5'-CCTCTTTGCTGCTTTCACACATG-3', IL8 for 5'-TTGGCAGCCTTCCTGATTTC-3', IL8 rev 5'-TGGCAAAACTGCACCTTCAC-3', monocyte chemoattractant protein (MCP) 1 for 5'-CTCGCTCAGCCAGATGCAATC-3', MCP1 rev 5'-AAGTTATAACAGCAGGTGACTGG-3', RANTES for 5'-CCAACCCAGCAGTCGTCTTTG-3', RANTES rev 5'-TGGCACACACTTGGCGGTTC-3'. miRNAs were quantified by stem-loop reverse transcription (RT)-PCR according to Chen *et al.* (179) using the following primers: miR-18a RT 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTATCT-3', miR-18a for 5'-GGCGGTAAGGTGCATCTAGT-3', miR-19 RT 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTT-3', miR-19a for 5'-CGGCGGTGTGCAAATCTATGC-3', miR-92a RT 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGGC-3', miR-92a for 5'-CGGCGGTATTGCACTTGTCCC-3', universal miR rev 5'-GTGCAGGGTCCGAGGT-3', miR-20a RT 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACCTG-3', miR-20a for 5'-CCTTAAAGTGCTTATAGTGCAG-3', miR-20a rev 5'-

TGCAGGGTCCGAGGTAT-3'. Data were analyzed by the comparative Ct method with GAPDH mRNA employed as endogenous control.

Plasmid construction

A part of the C13orf25 promoter (-1341 to +11, as described in (180)) was cloned into pGL3basic and the GAPDH promoter (-1087 to -24) was cloned into pRL (Promega). The 3' untranslated regions (3'UTRs) of dual-specific phosphatase 16 (DUSP16; 1-936 bp), protein tyrosine phosphatase type IV, member 3 (PTP4A3; 1-476bp) and TNF alpha-induced protein 3 (TNFAIP3; 1-1996bp) were cloned into pmirGLO (Promega). Site-directed mutagenesis PCR was used to mutate the NF-κB binding site (-959 to -950) in the C13orf25 promoter construct and the miR-18a seed matches in the 3'UTR constructs (DUSP16: 392-398bp; PTP4A3: 405-412bp; TNFAIP3: 671-677bp).

Transfection of small RNAs

RASF were transfected with 25nM precursor-miRNAs (pre-miR) or 100nM anti-miR-18a (both from Ambion) using Lipofectamin 2000 (Invitrogen). After 6h or 24h, the transfection mixture was replaced by fresh medium. Small interfering RNAs (siRNAs, 100nM; Qiagen) were transfected using the Primary Mammalian Fibroblast Nucleofection Kit (AMAXA/Lonza). Pre-miR Negative Control #1, anti-miR Negative Control #1 and AllStars Negative Control siRNA, respectively, served as transfection controls. 24h or 48h after transfection, cells were serum-starved for another 24h and stimulated with TNFα as indicated.

Reporter gene assay

Using nucleofector technology (AMAXA/Lonza), RASF were transfected with 1.2µg pRL_GAPDH and 1.8µg pGL3basic_C13orf25prom (wildtype or mutated), after 24h serum-starved for another 24h, and stimulated with TNFα (10ng/ml) for 8h. To measure NF-κB activity, RASF were transfected with 1.2µg pRL_GAPDH and 1.8µg pGL4.32 (five NF-κB response elements in row; Promega) by AMAXA Nucleofection. After 24h, RASF were transfected with pre-miR negative control #1 or pre-miR-18a using Lipofectamin 2000 as described above. 48h later, cells were serum-starved for 24h and stimulated with TNFα (10ng/ml) for 5h.

Using Lipofectamin 2000, Hek293 cells were transfected with 180ng pRL_GAPDH and 360ng pGL3basic_C13orf25prom (wildtype or mutated) and stimulated with TNFα (10ng/ml) for 4h and 8h. For the validation of miR-18a targets, Hek293 cells were transfected with 230ng pmirGLO_DUSP16, pmirGLO_TNFAIP3 or pmirGLO_PTP4A3 (wt or mutated miR-18a seed match) together with 25nM pre-miR negative control #1 or pre-miR-18a and harvested after 24h.

Firefly luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) and normalized to the activity of *Renilla* luciferase. An arbitrary cut-off of 1000 relative luciferase units was used for data analysis.

MMP-1, IL-6, IL-8, MCP-1 and RANTES ELISA

To measure MMP-1, IL-6, IL-8, MCP-1 and RANTES in cell culture supernatants commercially available ELISA development kits were used (MMP-1 and RANTES: R&D Systems; IL-6, IL-8 and MCP-1: BD Biosciences).

Isolation of peripheral blood leukocytes

Fresh blood from healthy donors was diluted with 1 volume isotonic NaCl solution and treated with 8 volumes hypotonic erythrocyte lysis buffer (155mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA, pH7.4) for 5 min on ice. After centrifugation, lysis was repeated with 4 volumes and then 1.5 volumes erythrocyte lysis buffer in isotonic NaCl. Cells were washed twice with ice-cold phosphate buffered saline (PBS), counted and resuspended in RPMI medium at a concentration of 10^7 cells/ml.

Leukocyte transwell migration assay

In 100µl volume, $1x10^6$ leukocytes per insert were seeded to a 96-well transwell plate (5µm polycarbonate membrane, Corning Costar). Medium (RPMI supplemented with 0.5% FCS) or conditioned medium (CM, 150µl per well) from pre-miR-18a or control transfected RASF was added to the feeder tray beneath the transwell. Leukocytes were allowed to migrate for 6h. To facilitate detachment of adhered cells, 50µl ice-cold 20mM EDTA/0.5% FCS in PBS was added to the lower wells and plates were incubated on ice for 15min. Migrated cells were counted with a CASY Cell Counter (Schärfe Systems). Leukocytes from two different healthy donors were used for analysis of each CM preparation and *vice versa*. All conditions were measured in duplicates.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot

For Western blot, rabbit-anti-TNFAIP3 (Abcam), mouse-anti-PTP4A3 (R&D Systems), and mouse-anti-α-tubulin (Sigma-Aldrich and Abcam) antibodies were used. Bands were detected utilizing horseradish peroxidase-labeled species specific secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence (GE Healthcare). Protein expression was quantified by spot densitometry with Alpha Imager software (Alpha Innotech) or Bio-1D software (Vilber Lourmat).

Statistics

For statistical analysis, GraphPad Prism 5.0 software was used. Data were tested for Gaussian distribution using the Shapiro-Wilk normality test and were analyzed by parametric (two-tailed paired t-test) or non-parametric (Wilcoxon matched-pairs signed rank test) statistical tests as appropriate. Values are presented as mean±standard deviation (SD). A p-value < 0.05 was considered significant.

4. Results

TNFα induces the expression of C13orf25 and mature miRNAs from the miR-17-92 cluster in RASF

TNFα is one of the major cytokines in the pathogenesis of RA (48, 54). Therefore we investigated whether TNFα could influence the expression of miR-17-92 in RASF. Figure 1A demonstrates that the primary transcript of miR-17-92, C13orf25, was induced by TNFα in a time-dependent manner. Accordingly, mature miRNAs derived from miR-17-92 – i.e. miR-18a, -19a, -20a and 92a – were upregulated at 8h and 16h of TNFα stimulation (Figure 1B).

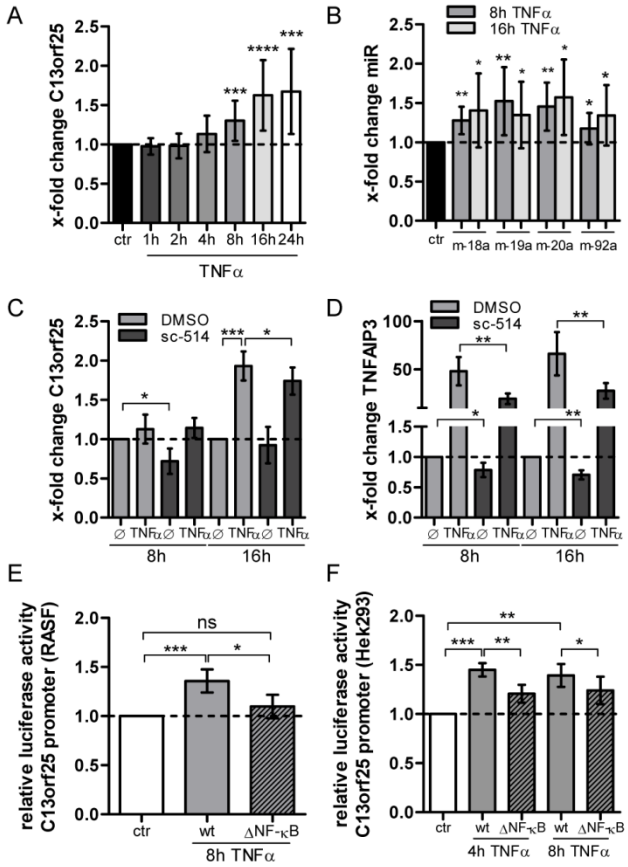

Figure 1 TNFα induces the expression of the miR-17-92 cluster *via* an NF-κB binding site in the C13orf25 promoter.
A. RASF (n=4-16) were stimulated with TNFα for the indicated time points. The C13orf25 primary transcript was significantly induced at 8h, 16h and 24h of stimulation. B. TNFα stimulation for 8h and 16h increased the levels of mature miRNAs - miR-18a, -19a, -20a and -92a – from miR-17-92 in RASF (n=15-16). C and D. RASF (n=5) were treated with sc-514 and stimulated with TNFα. sc-514 reduced the TNFα-induced induction of C13orf25 (at 16h; C) and TNFAIP3 (D). E and F. RASF (n=6; E) and Hek293 cells (n=5; F) were transfected with reportergene constructs encompassing the C13orf25 promoter with wildtype (wt) or mutated (ΔNF-κB) NF-κB binding site and stimulated with TNFα for 4h and/or 8h. C13orf25 promoter activity was induced in the wt construct whereas mutation of the NF-κB binding site inhibited the TNFα-mediated increase in C13orf25 promoter activity. Data are presented as mean±SD. ns=not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by paired t-test or Wilcoxon matched-pairs signed rank test.

The TNFα-induced C13orf25 expression depends on a NF-κB binding site in its promoter

TNFα is an important inducer of NF-κB and Jun kinase (JNK) signaling. To study whether C13orf25 induction by TNFα depends on these pathways, we treated RASF with sc-514, a chemical inhibitor of the Inhibitor of IκB kinase β which is upstream of NF-κB nuclear translocation. sc-514 partly inhibited TNFα-induced C13orf25 expression (Figure 1C). Figure 1D shows the expression of TNFAIP3, an established NF-κB target gene and negative regulator of NF-κB signaling in RASF (67, 181), which was used as positive control for effective inhibition of NF-κB signaling by sc-514. In

contrast, treating RASF with the JNK inhibitor SP600125 did not affect TNFα-induced C13orf25 expression (data not shown).

Zhou *et al.* previously demonstrated that the C13orf25 promoter contains three NF-κB binding sites of which the most proximal to the transcription start site (at -827bp) is responsible for LPS-induced C13orf25 transactivation in human biliary epithelial cells (182). We therefore addressed whether the TNFα–induced transcription of C13orf25 in RASF depends on this NF-κB binding site by utilizing a reportergene construct encompassing the C13orf25 promoter with wildtype or mutated NF-κB binding site controlling the firefly luciferase gene. The C13orf25 promoter activity was enhanced in RASF stimulated with TNFα for 8h and this induction was diminished when using the C13orf25 promoter construct in which the NF-κB binding site had been mutated (Figure 1E). Similar results were obtained when Hek293 cells were used for the reportergene assay (Figure 1F) suggesting that NF-κB-dependent induction of C13orf25 by TNFα is a general mechanism which might be applicable to other cell types than RASF. Since NF-κB blockade and NF-κB binding site mutation, however, did not completely abolish TNFα-dependent C13orf25 induction other pathways might be involved in this process as well.

Enforced expression of miR-18a upregulates MMP-1, IL-6, IL-8, MCP-1 and RANTES and increases the chemoattractive potential of RASF

A major hallmark of RASF is the production of matrix-degrading enzymes and inflammatory mediators, i.e. cytokines and chemokines, contributing to cartilage degradation and infiltration of the joint by immune cells (19). To elucidate which role miRNAs from the miR-17-92 cluster might play in the activation of RASF we chose the expression of MMP1 as a functional read-out for the effect of miR-17-92 transfection on RASF. We transfected RASF with precursor molecules of miR-18a, -19a, -20a and -92a (pre-miRs) (representing the four different miRNA families of the cluster) and subsequently stimulated with TNFα for 6h. Only pre-miR-18a transfection led to a significant increase in both the constitutive and the TNFα-induced expression of MMP1 mRNA (Figure 2A). Our further experiments thus were focused on this miRNA. To get a deeper insight into the role of miR-18a in the activation of RASF we measured MMP-1, IL-6, IL-8, MCP-1 and RANTES in pre-miR-18a- and anti-miR-18a-transfected RASF at the mRNA level (Figures 2B and 2C). The constitutive expression of MMP-1, IL-6, MCP-1 and RANTES was increased by pre-miR-18a transfection, and the TNFα-induced upregulation (at 24h stimulation) of MMP-1, IL-6, IL-8, MCP-1 and RANTES was further enhanced by pre-miR-18a transfection. Conversely, inhibition of miR-18a using anti-miR-18a significantly reduced the expression of IL8 and MCP1 under unstimulated conditions. In TNFα-treated cells, antagonizing miR-18a showed a trend towards reduced cytokine expression. These results, however, did not reach statistical significance. One can speculate that in TNFα-stimulated cells anti-miR-18a blocks miR-18a only in an inefficient manner (Figure 2D). In this regard, TNFα

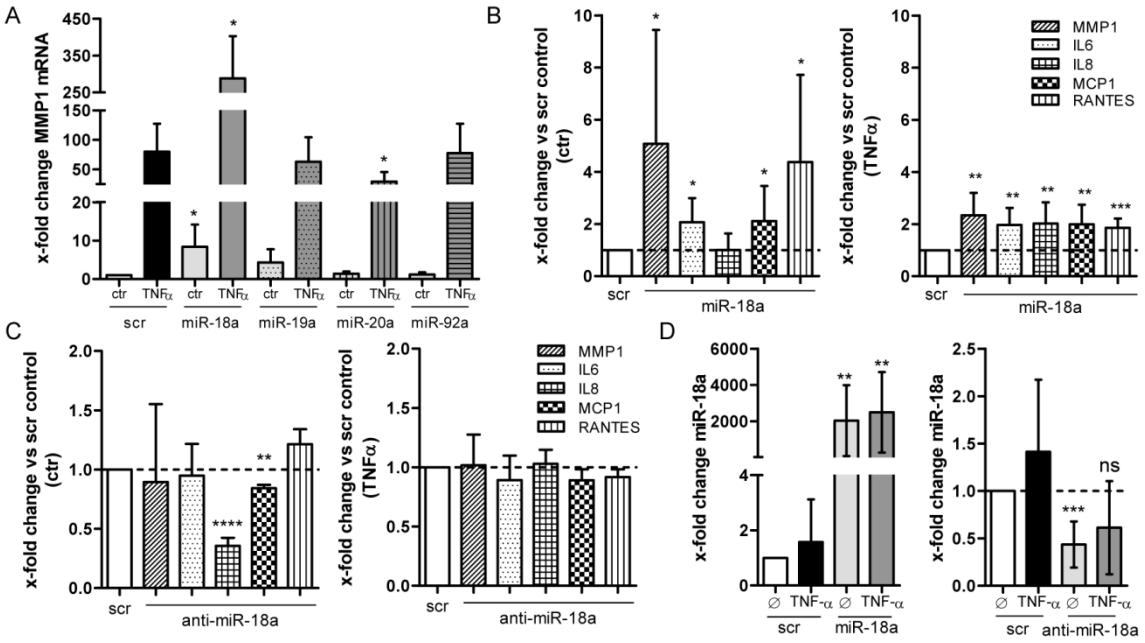

Figure 2 The miR-17-92-derived miR-18a promotes the expression of matrix-degrading enzymes and inflammatory mediators in RASF.

A. RASF (n=5) were transfected with precursor molecules of single miRs and stimulated with TNFα for 6h. B. RASF (n=8) were transfected with pre-miRs and after 48h stimulated with TNFα for 24h. mRNA levels of all studied genes were increased in pre-miR-18a-transfected RASF in unstimulated conditions (with the exception of IL8) and following TNFα stimulation. C. RASF (n=8) were transfected with anti-miR-18a and after 24h stimulated with TNFα for 24h. IL8 and MCP1 mRNA levels were reduced in unstimulated anti-miR-18a-transfected RASF. D. miR-18a was overexpressed (left) and silenced (right) in RASF studied in B and C. Data are presented as mean±SD. ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by paired t-test or Wilcoxon matched-pairs signed rank test.

seems to counteract the anti-miR-18-mediated inhibition of miR-18a by inducing the expression of miR-18a in RASF. Our data indicate that miR-18a contributes to the activation of RASF by upregulating the expression of important mediators of matrix infiltration and degradation.

To further corroborate our findings we measured secreted protein levels of MMP-1, IL-6, IL-8, MCP-1 and RANTES in the supernatants of RASF transfected with pre-miR-18a. Analogous to the mRNA data (Figure 2B), miR-18a-overexpressing RASF secreted higher levels of MMP-1 and the cytokines investigated than control cells (Figure 3A).To determine whether the effects of miR-18a on RASF gene expression, in particular the increased secretion of the chemokines IL-8, MCP-1 and RANTES, might indeed play a functional role in the pathogenesis of RA we performed a transwell migration assay using peripheral blood leukocytes from healthy donors and conditioned medium (CM) from RASF transfected with pre-miR-18a and stimulated with TNFα placed in the lower compartment as a chemoattractant. Figure 3B demonstrates that CM from pre-miR-18a-transfected and TNFα-stimulated RASF attracted more leukocytes towards the lower chamber than from scrambled control RASF. These experiments show that miR-18a upregulation in RASF increases the chemoattractive potential of these cells and, thus, might contribute to the infiltration of the inflamed joint with immune cells.

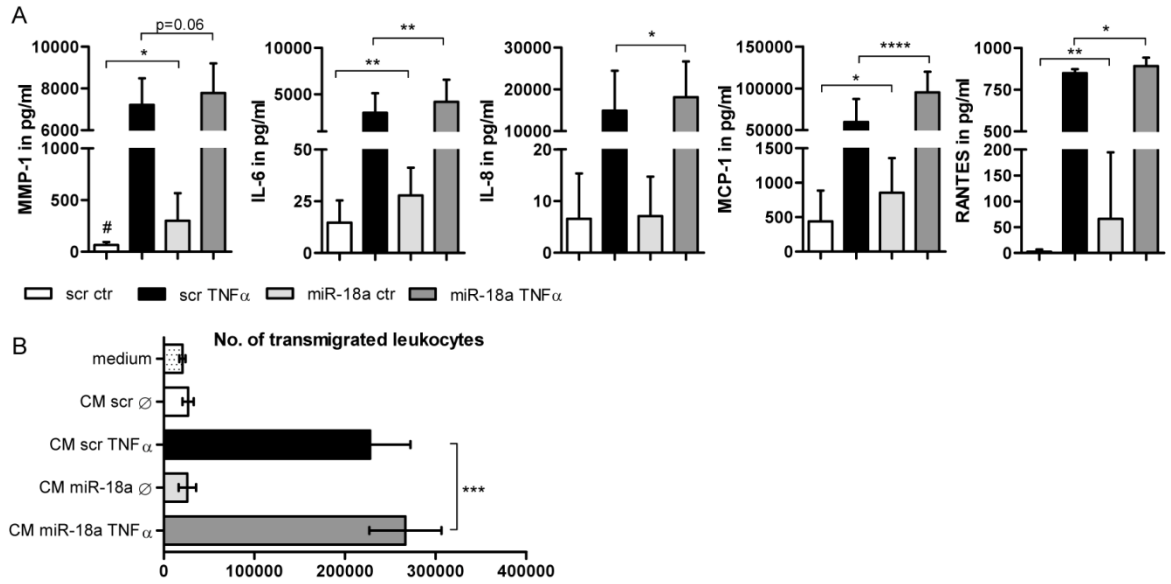

Figure 3 miR-18a confers increased chemoattractive potential to RASF.

A. Levels of MMP-1, IL-6 and of the chemokines IL-8, MCP-1 and RANTES were increased in the supernatants of pre-miR-18a-transfected RASF (n=8, same as in Figure 2B). # MMP-1 levels from unstimulated scr RASF were detected below the lowest standard value (156 pg/ml). B. In a transmigration assay, conditioned medium (CM) from pre-miR-18a-transfected and TNFα-stimulated RASF (n=8) attracted increased numbers of leukocytes towards the lower chamber. Leukocyte migration towards CM from unstimulated RASF was similar to background migration (i.e. medium only instead of CM). Data are presented as mean±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by paired t-test or Wilcoxon matched-pairs signed rank test.

miR-18a directly targets the NF-κB signaling inhibitor TNFAIP3 in RASF

In RASF, the expression of MMPs, cytokines and chemokines depends on the activation of important signaling pathways such as NF-κB, p38 and JNK signaling (183, 184). Based on the fact that miRNAs are negative regulators of gene expression while, on the other hand, we found miR-18a to upregulate downstream targets of TNFα signaling in RASF, we hypothesized that miR-18a might repress inhibitors of signal transduction pathways. Using the TargetScan database (www.targetscan.org) for computational prediction of miRNA targets we selected the NF-κB pathway inhibitor TNFAIP3 (181), the p38 phosphatase PTP4A3 (185) and the JNK phosphatase DUSP16 (186) as potential candidates for target validation. Since miRNAs repress gene expression by binding to the 3'UTRs of mRNAs leading to mRNA degradation and/or inhibition of translation (113, 177), we cloned the 3'UTRs of the three potential miR-18a targets into a reporter vector downstream of the luciferase gene. Co-transfection of these reporter vectors with pre-miR-18a into Hek293 cells reduced the luciferase activity when it was under control of the TNFAIP3 and PTP4A3 3'UTRs. Pre-miR-18a also reduced the DUSP16 3'UTR-controlled luciferase activity, however, without reaching statistical significance. Mutation of the respective miR-18 seed matches in those 3'UTRs rescued luciferase activity in miR-18a transfected cells (Figure 4A). These data indicate that miR-18a directly represses TNFAIP3 and PTP4A3 *via* the miR-18 seed matches in their 3'UTRs.

Subsequently, we analyzed the expression of TNFAIP3 and PTP4A3 in RASF transfected with pre-miR-18a. Whereas PTP4A3 mRNA levels were not affected TNFAIP3 mRNA expression was in-

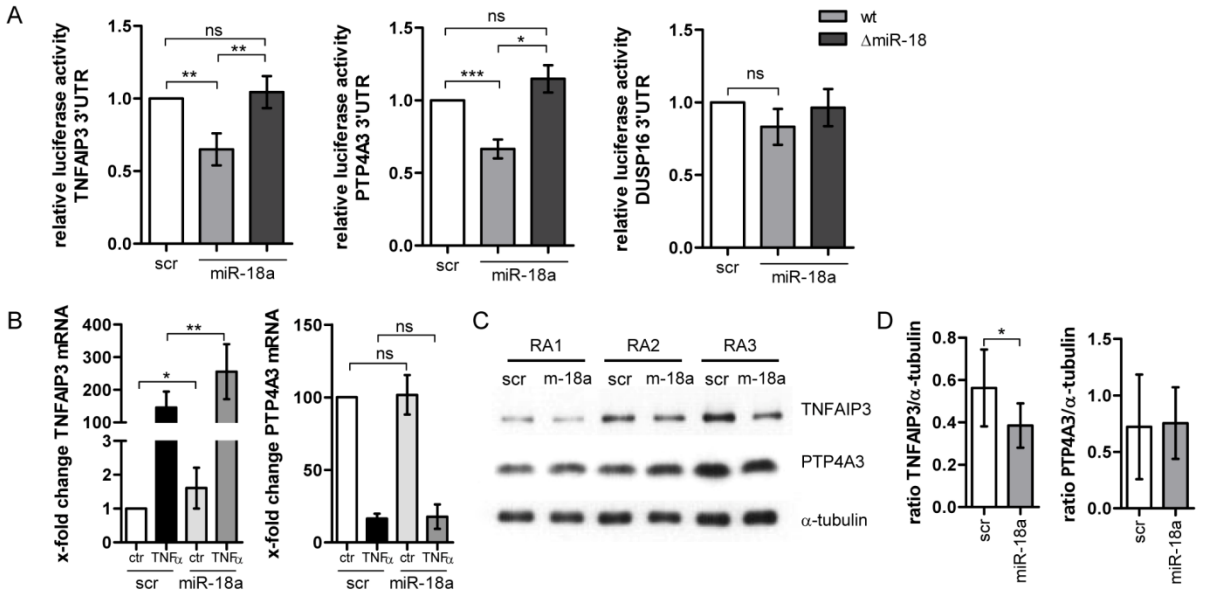

Figure 4 The NF-κB signaling pathway inhibitor TNFAIP3 is a direct target of miR-18a in RASF.

A. Utilizing reporter vectors encompassing the 3' untranslated regions (UTRs) of TNFAIP3, PTP4A3 and DUSP16, pre-miR-18a transfection significantly reduced the luciferase activity of the wildtype (wt) TNFAIP3 and PTP4A3 reporter constructs; mutated miR-18a seed matches (ΔmiR-18) rescued the luciferase activity back to levels of scr transfected cells (n=4 each). B. pre-miR-18a transfection increased TNFAIP3 mRNA levels whereas PTP4A3 mRNA levels were not influenced in RASF (n=8, same as in Figure 2B). C. Protein expression of TNFAIP3 was reduced in RASF (n=7) transfected with pre-miR-18a for 48h whereas PTP4A3 levels were not affected (quantification in D). Data are presented as mean±SD. ns=not significant, *p<0.05, **p<0.01, ***p<0.001 by paired t-test or Wilcoxon matched-pairs signed rank test.

creased in RASF overexpressing miR-18a (Figure 4B). Since miRNAs often only inhibit translation and do not mediate degradation of the target mRNA, we also measured protein levels of TNFAIP3 and PTP4A3 in miR-18a transfected RASF (Figures 4C and D). Despite directly targeting the PTP4A3 3'UTR in the reporter gene assay miR-18a transfection failed to reduce PTP4A3 levels in RASF. In contrast, TNFAIP3 protein levels were reduced at 48h after transfection. This discrepancy with the increased mRNA levels of TNFAIP3 might be explained by the fact that TNFAIP3 itself is a negative feedback regulator of NF-κB and, thus, translational repression of TNFAIP3 by miR-18a in RASF may induce the transcriptional activation of TNFAIP3 *via* enhancing NF-κB signaling.

Concomitant upregulation of C13orf25 and TNFAIP3 in TNFα-stimulated RASF

We next examined the expression of TNFAIP3 and C13orf25 in matched TNFα-stimulated RASF samples. Transcription of both TNFAIP3 and C13orf25 was induced by TNFα (Figures 5A and B). Interestingly, the significant upregulation of TNFAIP3 protein (Figures 5C and D) was much weaker than at the mRNA level suggesting that the efficiency of translation was reduced under these conditions. This may, at least in part, be due to the concomitant upregulation of miR-17-92, in particular of miR-18a, and thus simultaneous translational repression of TNFAIP3. A similar scenario has been suggested previously for the relationship of c-Myc-induced miR-17-92 and its target E2F leading to diminished induction of E2F protein compared to the corresponding mRNA (178).

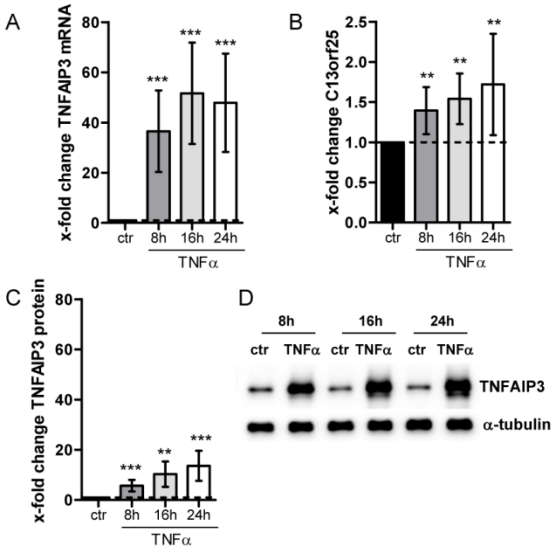

Figure 5 Expression kinetics of TNFAIP3 and C13orf25 in TNFα-stimulated RASF.

RASF (n=8) were stimulated with TNFα for 8h, 16h and 24h. mRNA levels of TNFAIP3 (A), C13orf25 (B) and protein levels of TNFAIP3 (C, representative Western blot image in D) were induced by TNFα. Data are presented as mean±SD. **p<0.01, ***p<0.001 by paired t-test or Wilcoxon matched-pairs signed rank test.

Silencing of TNFAIP3 mimics the miR-18a-mediated effects in RASF

Having demonstrated that TNFAIP3 is a direct target of miR-18a in RASF, we addressed whether silencing of this NF-κB inhibitory gene could mimic the effects of miR-18a on MMP1 and cytokine expression. Therefore we transfected RASF with siRNAs targeting TNFAIP3, stimulated the cells with TNFα and measured target genes by real-time PCR. Figure 6A demonstrates that TNFAIP3 silencing could be achieved efficiently. Under unstimulated conditions, silencing of TNFAIP3 in-

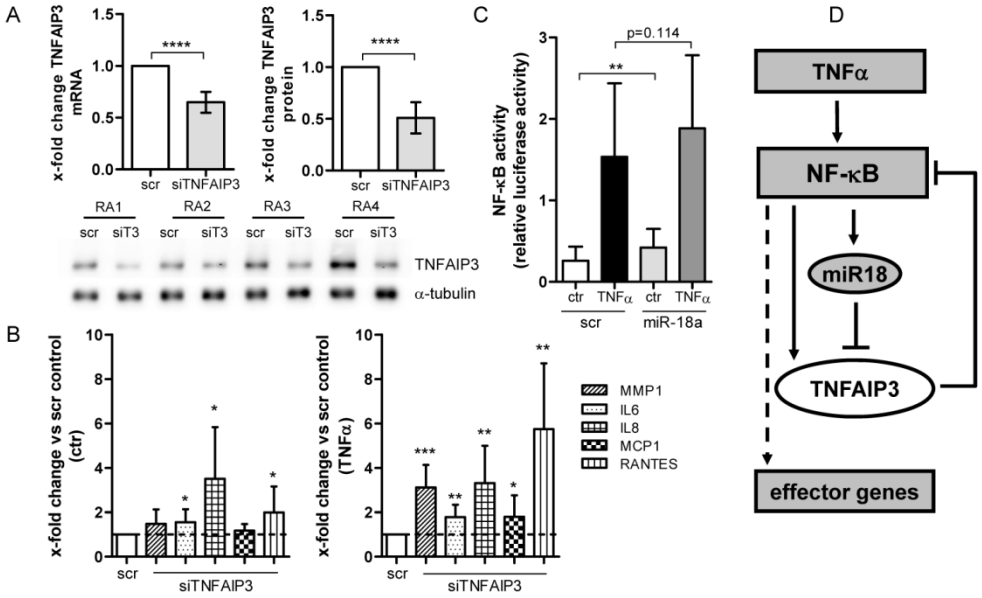

Figure 6 The TNFα-induced miR-18a enhances RASF activation through a positive feedback loop in NF-κB signaling *via* TNFAIP3.

A and B. RASF (n=8) transfected with siRNA targeting TNFAIP3 were stimulated with TNFα for 24h. A. TNFAIP3 expression was efficiently silenced. B. Silencing of TNFAIP3 increased constitutive levels of IL6, IL8 and RANTES mRNA while the TNFα-induced expression of all five genes studied was enhanced. C. RASF (n=8) transfected with a NF-κB responsive reporter vector and pre-miR-18a showed stronger activation of NF-κB signal transduction than scr-transfected RASF. Data are presented as mean±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by paired t-test or Wilcoxon matched-pairs signed rank test. D. Model of the signaling events in RASF involving miR-18.

creased the mRNA levels of IL6, IL8 and RANTES whereas MMP1 and MCP1 mRNA levels were not significantly affected. In contrast, under TNFα stimulation, silencing of TNFAIP3 increased the expression of all five genes studied (Figure 6B).

The TNFα-induced activation of NF-κB signaling is enhanced by miR-18a

Finally, we sought to investigate whether miR-18a could influence NF-κB signaling in RASF as suggested by the repression of the NF-κB pathway inhibitor TNFAIP3. As expected, RASF transfected with miR-18a displayed a stronger constitutive as well as TNFα-induced NF-κB activity (Figure 6C).

Taken together, we suggest the following model of the signaling events involving miR-18a in RASF (Figure 6D). Chronic synovial inflammation as simulated *in vitro* by TNFα induces activation of NF-κB and, in turn, upregulation of miR-18a and TNFAIP3. In a negative feedback loop, TNFAIP3 terminates the action of NF-κB. Repression of TNFAIP3 by miR-18a interferes with this feedback loop resulting in enhanced expression of downstream effector genes.

5. Discussion

In this work we have shown that i) the miR-17-92 cluster is induced in RASF by TNFα through activation of NF-κB, and ii), that miR-18a is involved in the upregulation of both constitutive and TNFα-induced secretion of MMP1 and inflammatory cytokines and chemokines and, thus, increases the chemoattractive potential of RASF. Moreover, iii) using reporter gene assays we have identified TNFAIP3 as a novel direct target of miR-18a and we could demonstrate that iv) by repressing TNFAIP3 expression miR-18a enhances NF-κB signaling in RASF. In conclusion, miR-18a constitutes a novel positive feedback loop of the NF-κB signaling pathway *via* translational repression of TNFAIP3 (Figure 6D) which might further aggravate the activated phenotype of SF in the pathogenesis of RA.

In recent years it has become clear that miRNAs are dysregulated in RA and, acting as repressors of gene expression, contribute to autoimmunity and joint destruction (reviewed in (176)). The miR-17-92 cluster has been associated with important inflammatory processes such as the acute-phase response (145) and maturation of the immune system. Xiao and colleagues, for example, have shown that overexpression of miR-17-92 in lymphocytes of mice led to the development of autoimmunity reflected by autoantibody production and lymphocytic infiltrates into nonlymphoid tissues (171). In a mouse model of systemic lupus erythematosus miR-17-92 was found to be specifically upregulated in splenic T cells (187) and another study showed that miR-17 and miR-19b are critical regulators of Th1 responses and Treg differentiation (188). Here we found an upregulation of the miR-17-92 primary transcript and its mature miRNAs in response to TNFα, which, in turn, repressed the NF-κB pathway inhibitor TNFAIP3 thus mediating proinflammatory functions in our

experiments. A recent study, on the other hand, found miR-19a and -19b to be repressed in RASF by stimulation with Toll-like receptor (TLR)2 and TLR4 ligands; moreover, TLR2 was identified as a direct target of the miR-19 family (189). Thus it may be assumed that different signals connected to the pathogenesis of RA might lead to a differential expression and function of miR-17-92 in RASF. Interestingly, miR-19a, similar to miR-18a, is predicted to target TNFAIP3 (www.targetscan.org) and our own unpublished data using reporter gene assays with the TNFAIP3 3'UTR confirmed this prediction. Furthermore, miR-19 was shown to repress the NF-κB inhibitor CYLD (190) and Gantier and colleagues very recently found that miR-19b does not only target TNFAIP3 but also other negative regulators of NF-κB signaling (191). Based on these findings we strongly argue for a proinflammatory role of miR-17-92 in the pathogenesis of RA.

In our screening to detect a functional role for miR-17-92 in RASF we chose MMP1 expression as read-out and found that miR-18a had significant effects on MMP1 mRNA levels; further analysis showed that miR-18a also enhances the expression of other important mediators involved in the pathogenesis of RA, including IL-6, IL-8, MCP-1 and RANTES. Based on these findings together with data from our previous work showing that miR-18a targets the signal transducer and activator of transcription (STAT)3 signaling inhibitor PIAS3 (protein inhibitor of activated STAT, 3) (145) we postulated that miR-18a similarly might target inhibitors of TNFα-induced signaling pathways and found, by a computational approach using TargetScan and subsequent validation experiments, that miR-18a acts as a repressor of TNFAIP3. Interestingly, it has been shown that knockout of TNFAIP3 in myeloid cells triggers the development of an erosive polyarthritis (66); conversely, adenoviral delivery of TNFAIP3 improved inflammation and bone destruction in collagen-induced arthritis (67). These data show that the NF-κB signaling pathway is a crucial mediator of arthritis and imply that controlling NF-κB activity (directly *via* TNFAIP3 or indirectly through miR-18a) in the different cell types involved in the pathogenesis of RA may be a promising therapeutic approach.

Beside TNFAIP3 we also identified PTP4A3 as a new target of miR-18a by reporter gene assay. However, pre-miR-18a transfection did not reduce PTP4A3 levels in RASF. Generally, target repression by miRNAs through the miRNA-induced silencing complex may be additionally regulated by RNA-binding proteins that facilitate or inhibit the miRNA-mRNA interaction depending on different cellular and environmental conditions (110). One might speculate that the functional discrepancy observed between PTP4A3 3'UTR targeting by miR-18a in Hek293 and in RASF result from the different cellular environment. In conclusion, it appears that repression of TNFAIP3 is the major contributor to the miR-18a-mediated upregulation of MMP1 and inflammatory cytokines. Silencing of TNFAIP3, however, did not copy the effects of miR-18a transfection completely as shown strikingly by experiments on IL8. miR-18a increased the expression of IL8 only when cells were stimulated with TNFα, whereas silencing of TNFAIP3 additionally upregulated the constitutive IL8 mRNA expression. These results implicate that additional, yet unidentified targets of miR-18a may be responsible for the final output in gene expression observed after pre-miR-18a transfection.

One such target may be connective tissue growth factor, which was shown to be de-repressed by anti-miR-18a treatment in glioblastoma spheroid cultures (192) and which has been connected to IL-6 and IL-8 expression in tendon fibroblasts (193). The involvement of connective tissue growth factor in MMP1 and cytokine expression in RASF remains elusive for the moment and warrants further experimental address.

We showed here for the first time that miR-18a is part of a positive regulatory loop in NF-κB signaling in RASF. Work from recent years has established a complex network of miRNAs positively and negatively regulating NF-κB signaling at different levels of the signaling cascade (reviewed in (194)). Two of those miRNAs that have been associated with the pathogenesis of RA are miR-146a and miR-155, of which the latter one is involved in the production of proinflammatory cytokines and the development of collagen-induced arthritis (122, 127, 129, 140). They are both induced through NF-κB-dependent pathways, but seem to be engaged in negative feedback loops. MiR-155, for instance, may repress inflammatory signaling by targeting the inhibitor of κB kinases β and ε (138). Together, these data support the importance of an intact NF-κB signaling; different levels of regulation appear to include the involvement of various miRNAs to result in a finely balanced activation pattern that becomes disturbed under pathological conditions such as RA.

In conclusion, together with our previous data (145) this work underscores the role of miR-18a as a regulator of intracellular signaling pathways that acts as an endogenous amplifier of extracellular signals. Further work is needed to complete the picture of miR-18a function in cell signaling and to unravel in more detail the role of miR-17-92 in autoimmune diseases, particularly in RA. Nevertheless, we have shown that miR-18a is induced by TNFα and is part of a positive feedback loop in the NF-κB signaling pathway in RASF; miR-18a thus increases MMP and cytokine expression and enhances the chemoattractive potential of RASF by potentiating the effect of TNFα. These data suggest that miR-17-92 plays an important role in TNFα-mediated signaling mechanisms which, although not directly evidenced by our experiments, might result in RASF-mediated cartilage destruction and immune cell infiltration of the joint in the pathogenesis of RA.

Acknowledgements

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CHAPTER IV

Conclusion and outlook

1. Long noncoding RNAs and epigenetic regulation in RASF

Although only about 2% of the genetic material codes for protein practically the entire genome is transcribed making up a large pool of noncoding RNA (71). Small noncoding RNAs include miRNAs, snRNAs, snoRNAs, tRNAs and piwi-interacting RNAs, each of them with well-established functions in gene regulation. Long noncoding RNAs (lncRNAs) have only recently been recognised as regulators of gene expression instead of being genetic junk (73). In the context of this doctoral thesis one lncRNA appeared to be of special interest, the HOX transcript antisense intergenic RNA (HOTAIR). In an attempt to elucidate the mechanisms of HOTAIR-mediated chromatin remodelling Tsai and colleagues identified epigenetic binding partners of HOTAIR, namely EZH2 and the lysine specific demethylase 1 (LSD1, an enzyme demethylating H3K4) (195). Folding into extensive secondary structures the ~2200 nt-long HOTAIR functions as a modular scaffold that co-ordinately localises EZH2 and LSD1 to target chromatin regions causing loss of the activating H3K4me3 mark and, simultaneously, gain of the silencing H3K27me3 mark (195). Similar to EZH2, HOTAIR is found overexpressed in many human tumours (196) and was shown to promote metastasis by targeting EZH2 to a certain set of genes inducing gene expression changes that favoured an invasive behaviour of cancer cells (197).

Although expression and function of HOTAIR and other lncRNAs are currently intensively studied in the field of cancer research nothing is known about them in autoimmune diseases and specifically RA. Hence, in a follow-up project to our previous findings concerning the role of EZH2 in RASF (**Chapter II**, (198)) we wanted to investigate whether HOTAIR (in cooperation with EZH2) might contribute to epigenetic dysregulation in RASF, which, in turn, might promote the activated phenotype of these cells.

The first data of this on-going project demonstrated that the expression of HOTAIR was strongly reduced in RASF (Figure 1A). Mimicking the inflammatory milieu to which synovial cells are exposed within the joint of RA patients, OASF stimulated with TNFα and IL-1β showed significantly decreased levels of HOTAIR compared with unstimulated control cells (Figure 1B). Based on the fact that a) EZH2 is upregulated in RASF, b) TNFα stimulation induces the expression of EZH2 (198) and c) HOTAIR is encoded in the *HOXC* gene cluster which is a prime target of EZH2-mediated gene regulation (72) we hypothesised that HOTAIR expression in SF might be regulated by EZH2 and H3K27me3. Overexpression of EZH2 in OASF significantly reduced HOTAIR levels (Figure 1C) and ChIP analysis revealed stronger H3K27 trimethylation at the *HOTAIR* promoter in RASF (Figure 1D). Accordingly, H3K27me3 levels at the *HOTAIR* promoter correlated with the ΔCt values of HOTAIR expression, i.e. the more H3K27me3 was present at the promoter the less it was expressed (Figure 1E). Together, these data show that the lncRNA HOTAIR is silenced in RASF due to increased EZH2-transferred H3K27me3 mark in its promoter.

In a next step, we wanted to illuminate the functional consequences of HOTAIR silencing in RASF. To this end OASF were transfected with siRNA targeting HOTAIR and the expression of mul-

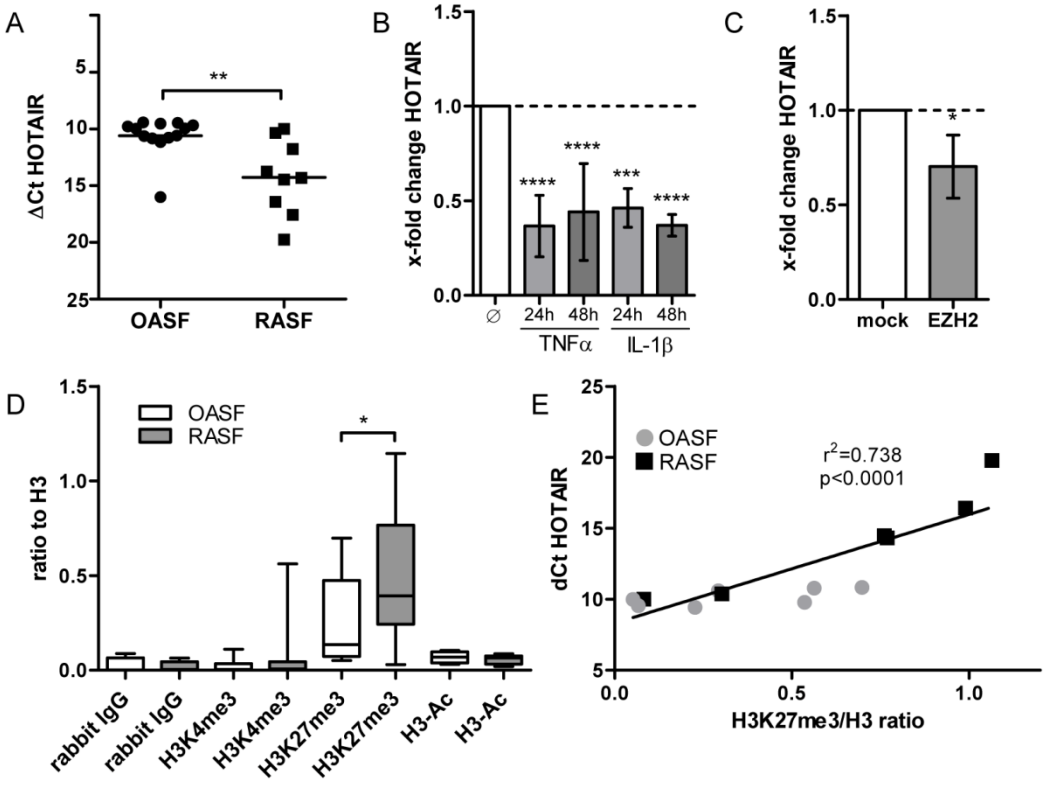

Figure 1 Epigenetic repression of the lncRNA HOTAIR in RASF.

A. HOTAIR expression is 12.7-fold stronger in OASF (n=13) than in RASF (n=9). B. TNFα- (n=11) and IL-1β- (n=5) stimulation reduced the expression of HOTAIR in OASF. C. Transfection of EZH2 silenced the expression of HOTAIR in OASF (n=4). D. The promoter of HOTAIR shows increased H3K27me3 levels in RASF (n=16) as compared with OASF (n=12). E. The expression of HOTAIR correlates with the promoter H3K27me3 silencing mark occupancy.

tiple genes involved in the pathogenesis of RA was analysed. mRNA levels of the MMPs 1, 3, 13 and 14 were significantly increased upon silencing of HOTAIR (Figures 2A, B, C and D). Silencing of HOTAIR furthermore increased the TNFα-stimulated induction of MMPs 1, 3 and 13 whereas no sig-

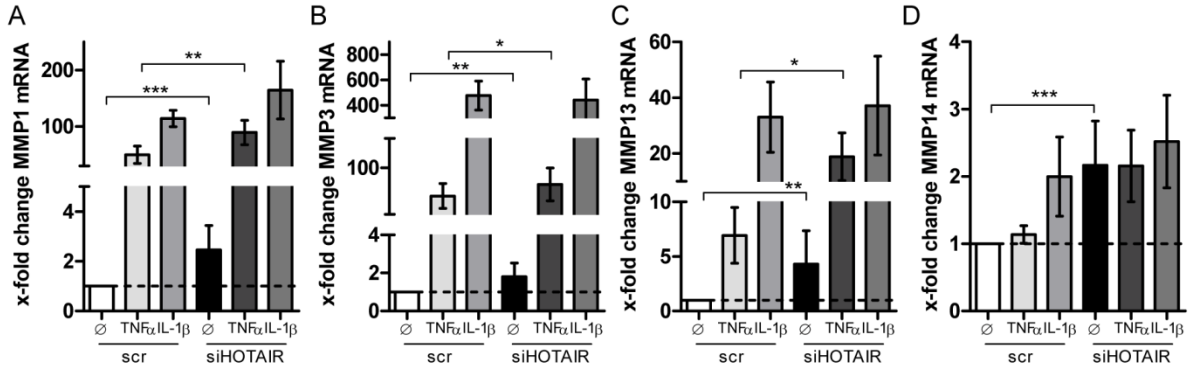

Figure 2 HOTAIR silencing increases the expression of matrix-degrading enzymes.

OASF (n=11) were transfected with siRNA targeting HOTAIR (siHOTAIR) and stimulated with TNFα or IL-1β (n=5). The expression of MMP1 (A), MMP3 (B), MMP13 (C) and MMP14 (D) was increased upon HOTAIR silencing.

nificant difference was seen following stimulation with IL-1β (Figure 2). These data imply that HOTAIR might influence TNFα-induced but not IL-1β-induced signalling pathways in RASF.

Additional experiments are now planned to confirm the effect of HOTAIR silencing on the expression of MMPs at the protein level and to further elucidate the mechanisms of HOTAIR regulation and HOTAIR function in SF.

Concerning the regulation of HOTAIR, EZH2 expression in RASF will be silenced using siRNA, cells will subsequently be stimulated with TNFα and IL-1β and the expression of HOTAIR will be analysed. Chromatin from SF will furthermore be assessed for the H3K27me3 levels at the *HOTAIR* promoter before and after TNFα and IL-1β stimulation using ChIP. These experiments will clarify whether TNFα- and IL-1β-mediated repression of HOTAIR depends on the action of EZH2.

To determine the expression of HOTAIR *in vivo*, *in-situ* hybridisation will be performed on RA and OA synovial tissue slides. It will further be of interest to address the subcellular localisation of HOTAIR; therefore, fluorescence *in-situ* hybridisation and confocal microscopy will be performed using cultured SF.

To better understand how HOTAIR might regulate MMP expression reportergene assays to study the activity of the NF-κB and AP-1 signalling pathways will be carried out using RNA interference with EZH2 and HOTAIR in RASF and OASF, respectively. RNA immunoprecipitation experiments will illuminate a possible functional interaction of EZH2 with HOTAIR in SF. SF stimulated with TNFα and IL-1β will be analysed for their H3K27me3 levels at the MMP promoters. To see whether HOTAIR can directly affect the MMP promoter regions further ChIP analyses for H3K27me3 will be carried out in HOTAIR-silenced SF. Alternatively, if the MMP promoter epigenetic marks are not influenced by manipulating cellular HOTAIR levels possible HOTAIR targets may be found in the signalling pathways upstream of MMP induction (i.e. NF-κB and AP-1 pathways).

Altogether, this project will complement the data from **Chapter II** (198) further highlighting the importance of epigenetic gene regulation, and of EZH2 in particular, in the activated phenotype of RASF. It will furthermore clarify whether lncRNA dysregulation may not only affect malignant diseases but also other pathologic conditions such as RA. Thus, this work will contribute to a better understanding of the epigenome of RA.

2. EZH2 and miR-17-92 in RASF and the big picture

During my doctorate I have investigated expression and function of a histone methyltransferase and a miRNA cluster in the activated phenotype of RASF. In recent years it has become clear that the pathogenesis of RA is accompanied by epigenetic dysregulation and differences in the expression of miRNAs. Although it is not known yet whether these changes in gene regulation are the cause or the consequence of RA it is nevertheless crucial to understand these mechanisms to be able to develop

better and targeted therapies in the future. Thus, this work has added new knowledge to a novel and exciting field of research substantiating the concept of epigenetic dysregulation and the importance of miRNA networks in the activated phenotype of RASF.

In **Chapter II** we could show that (i) the histone methyltransferase EZH2 is upregulated in RASF, (ii) inducible by TNFα which was dependent on the NF-κB-, JNK- and E2F-Rb pathways, and that (iii) the Wnt signalling inhibitor SFRP1 is a target of EZH2-mediated gene silencing in RASF (198). In a follow-up project we are currently extending those data by showing that EZH2 also targets a lncRNA – HOTAIR – for silencing in RASF (Figure 3).

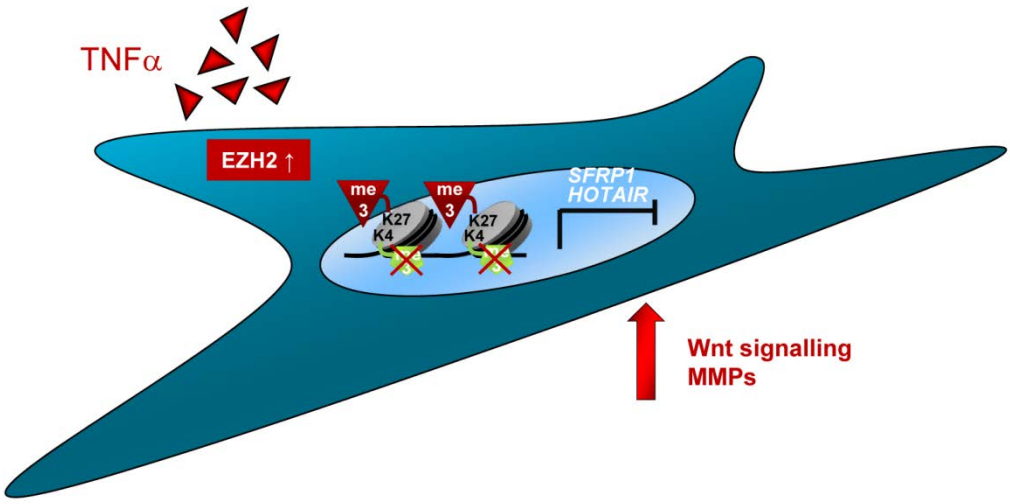

Figure 3 The role of EZH2 in the activated phenotype of RASF

EZH2 is overexpressed in RASF and is further induced by TNFα. EZH2 overexpression leads to repression of SFRP1 and HOTAIR and the gene promoters of *SFRP1* and *HOTAIR* display elevated levels of the repressive H3K27me3 mark correlating with their decreased expression. TNFα stimulation further inhibits the expression of SFRP1 and HOTAIR implying a possible connection to TNFα-induced EZH2 upregulation. The repression of SFRP1 insinuates increased Wnt signalling which, as shown previously, affects the expression of cytokines, MMP3 and extracellular matrix molecules in RASF (163, 164). Silencing of HOTAIR results in the upregulation of MMPs.

In **Chapter III** we studied the miR-17-92 cluster and specifically the miR-17-92-derived miR-18a and found it to be involved in a positive feedback loop in NF-κB signalling in RASF (199). We demonstrated that (i) miR-17-92 is induced upon stimulation with TNFα in a NF-κB-dependent manner and (ii) miR-18a enhances MMP and inflammatory mediator production by RASF. (iii) Validating the NF-κB inhibitor TNFAIP3 as a novel direct target of miR-18a and (iv) showing enhanced NF-κB signalling upon pre-miR-18a transfection in RASF we uncovered a new mechanism of regulating NF-κB activity as miR-18a interferes with the negative feedback loop of NF-κB-dependent TNFAIP3 induction (Figure 4).

At present, no connection is known between EZH2 and miR-17-92. Although miR-17-92 may be regulated by epigenetic mechanisms EZH2-mediated H3K27 methylation at the C13orf25 promoter has not been studied yet. During Mϕ differentiation, miR-17-92 was shown to be epigenetically re-

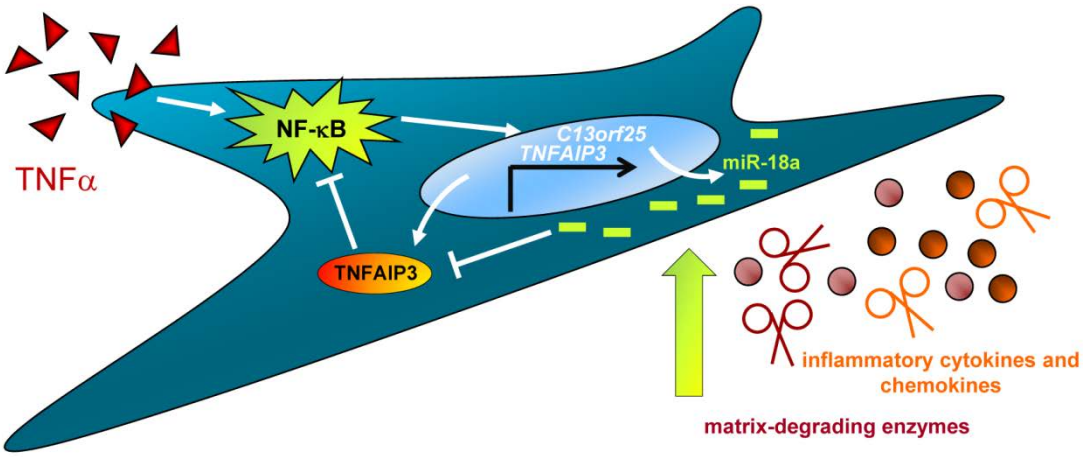

Figure 4 miR-18a is part of a positive feedback loop in NF-κB signalling in RASF

The NF-κB signal transduction pathway is activated upon stimulation with TNFα which induces the expression of inflammatory mediators and matrix-degrading enzymes. TNFα also upregulates TNFAIP3 acting as an endogenous inhibitor of NF-κB signalling. The concomitant induction of the miR-17-92 cluster (C13orf25), in particular of miR-18a, interferes with this negative feedback loop by the repression of TNFAIP3. The resulting increase in NF-κB activity leads to an enhanced expression of cytokines and MMPs.

pressed by the recruitment of a histone demethylase, Jarid1b, and the resulting loss of the H3K4me3 activating mark (200). In an ACR Abstract 2011 we could demonstrate that miR-196a is repressed in RASF due to increased H3K27me3 and decreased H3K4me3 in its promoter (201) thus highlighting that EZH2 may indeed regulate miRNAs in SF. The 3'UTR of EZH2 contains miRNA binding sites and several miRNAs (miR-26a, miR-101, miR-124) have been validated as direct regulators of EZH2 (miRecords http://miRecords.umn.edu/miRecords (202), October 2012). Of interest, miR-124a is downregulated in RASF (123) which might contribute to the upregulation of EZH2 in RASF. Future work will have to address the epigenetic mechanisms regulating miR-17-92 and the miRNA-mediated mechanisms regulating EZH2 in RASF to fully elucidate how these pathways might be intertwined.

The aim of translational research as performed during this doctorate should ultimately be the transfer to a clinical application, diagnostic or therapeutic. With respect to the roles of EZH2 and miR-18a in the activated phenotype of RASF both represent potential therapeutic targets. However, the data presented here refer to RASF only and more research is indispensable to determine the function of both targets in other cell types/compartments involved in the pathogenesis of RA.

miRNAs may be targeted *in vivo* by the use of antagomiRs, antisense miRNAs with stabilising modifications of the sugar phosphate backbone and a cholesterol moiety at the 3' end for better bioavailability (203). We have previously used antagomiR-20a to successfully prevent vascular remodelling and right heart hypertrophy in the hypoxic mouse model of pulmonary hypertension (144). Based on our data that miR-18a regulates essential inflammatory signal transduction pathways such as STAT3 (145) and NF-κB signaling (199) it might be interesting to test antagomiR-18a in an animal model of an inflammatory disease such as RA. Since the mentioned pathways are not just involved in RASF activation but also in other cell types important for the pathogenesis of RA (e.g.

monocytes, Mϕ, osteoclasts) antagomiR-18a might be an attractive tool of affecting all cellular compartments of RA simultaneously. Whether targeting miRNAs will be suitable for the treatment of human diseases is still an open question. So far, only one miRNA-based treatment strategy has entered phase II clinical trials – a miR-122 inhibitor for the treatment of Hepatitis C ("miravirsen", www.santaris.com). As with every other drug the consequences of systemic delivery of such a miRNA-based therapy must be carefully examined (204). Since miR-122 is expressed only in hepatic tissue antagonising miR-122 with miravirsen may cause less problems of unwanted effects in non-target organs, whereas targeting non-organ-specific miRNAs may be associated with serious adverse events. At the same time, the bioavailability of miRNA-based drugs is a problem. In comparison to small molecule inhibitors an antagomiR is much larger and delivering it to peripheral tissues such as the joint may prove challenging, a problem that might be overcome with intra-articular injections.

Therapeutic targeting of epigenetic mechanisms has already progressed further than miRNA-targeting therapy. Some drugs inhibiting DNA methylation and histone acetylation are yet FDA approved for the treatment of several haematologic malignancies (71, 79). Although an inhibitor for H3K27 methylation has been known for several years (5'-deazaneplanocin [DZNep]) its mode of action depends on the depletion of PRC2 components rather than direct inhibition of the EZH2-methylating activity (205). Furthermore, it was shown to inhibit histone methylation globally thereby not only decreasing H3K27me3 levels but also reducing H3R2me2, H3K4me3, H3K9me1, H3K9me2, H3K79me3 and H4K20me3 (206). The development of specific inhibitors to the enzymes transferring and removing the H3K27 methylation marks has only been reported this year. A recent study reported the discovery of a specific inhibitor of EZH2, EPZ005687, with ~50-fold selectivity over the closely related EZH1 protein and over 500-fold selectivity against 15 other protein methyltransferases (207). Although EPZ005687 only proved effective in inhibiting cell growth and inducing apoptosis in lymphoma cell lines harbouring activating EZH2 mutations it also reduced global H3K27me3 levels in wildtype cell lines (207). Similar results were obtained with the SAM-competitive GSK126 that showed more than 1000-fold selectivity over 20 other methyltransferases and was even 150-fold less potent towards EZH1 as compared with EZH2 (208). Furthermore, GSK126 efficiently inhibited the growth of subcutaneous xenografts of diffuse large B cell lymphoma cell lines and increased the overall survival of mice (208). These drugs might therefore represent useful tools to further study the importance of H3K27me3 in settings where EZH2 is upregulated and constitute a good basis for the development of better wt-EZH2 inhibitors. A small-molecule inhibitor GSK-J1 was found to specifically bind to and inhibit the two jumonji family H3K27 demethylases Jmjd3 and UTX (209). Treatment of LPS-stimulated human primary Mϕ with the GSK-J1 pro-drug GSK-J4 partially inhibited inflammatory gene expression and, moreover, blocked the LPS-induced loss of H3K27me3 at the *TNFA* promoter. Most importantly, it also impeded TNFα production from RA patient-derived Mϕ (209). Thus, while several lines of evidence argue for possible beneficial effects of inhibiting

H3K27 demethylation in inflammatory conditions such as RA, in particular regarding the role of Jmjd3 in the inflammatory gene expression of Mϕ (153, 209, 210), others argue for the opposite. Our own data demonstrate the upregulation of EZH2 in RASF, constitutively and upon TNFα stimulation (198). EZH2 was further shown to positively regulate NF-κB activity in estrogen receptor-negative breast cancer most likely due to a direct interaction with the NF-κB components RelA and RelB (211). Considering the fact that in RA the balance of bone resorption (osteoclasts) and formation (osteoblasts) is skewed towards resorption (27) it is interesting to note that the differentiation of osteogenic precursor cells depends on a loss in EZH2 activity which induces osteoblast-specific gene expression (212) and, thus, EZH2 inhibition might favour osteoblastogenesis. Furthermore, EZH2 was demonstrated to be a critical regulator of tumour angiogenesis being overexpressed in the vasculature of ovarian cancer and controlling endothelial cell gene expression (213), tube formation (214), migration and invasion (215). Since it is inducible by proangiogenic mediators such as VEGF (213) and FGF2 (214), molecules that are abundantly expressed in the RA synovium (35), EZH2 might be involved in the pathological synovial angiogenesis of RA. Taken together, it appears that H3K27 methylation is dysregulated under inflammatory conditions such as RA but the direction in which the balance is shifted may be dependent on the cell type studied. Therefore, future *in vitro* as well as *in vivo* studies using specific inhibitors of EZH2 and Jmjd3/UTX must shed more light on the involvement of this epigenetic mark in the pathogenesis of RA and in regulating gene expression in all cellular compartments of the RA joint.

In summary, with this doctoral thesis I have for the first time addressed histone methylation and the miR-17-92 cluster in the activated phenotype of RASF. We provided data showing that upregulation of EZH2 led to the epigenetic silencing of the tumour suppressor SFRP1 and the lncRNA HOTAIR contributing to the activation of RASF. Validating the miR-17-92-derived miR-18a as a novel direct regulator of TNFAIP3 we could identify a new positive feedback loop in the TNFα-NF-κB pathway enhancing inflammatory signalling in RASF.

The better understanding of epigenetic mechanisms and miRNA regulation in the inflammatory and destructive processes of RA will eventually help to establish the basis for the improvement of current treatment strategies and/or the development of novel targeted therapies in the future.

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Appendix

Abbreviations

ACPA	anti-citrullinated protein antibodiy
ACR	American College of Rheumatology
AP-1	activator protein 1
bp	basepairs
BSA	bovine serum albumin
CD	cluster of differentiation
ChIP	chromatin immunoprecipitation
CIA	collagen-induced arthritis
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DAMP	danger- or damage-associated molecular pattern
DKK1	Dickkopf-1
DMARD	disease-modifying antirheumatic drug
DMEM	Dulbecco's modified Eagle's medium
DNA	desoxyribonucleic acid
DNMT	DNA methyltransferase
DUSP16	dual-specific phosphatase 16
ECM	extracellular matrix
EED	embryonic ectoderm development
ELISA	enzyme-linked immunosorbent assay
EULAR	European League against Rheumatism
EZH2	Enhancer of Zeste homologue 2 (Drosophila)
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDAC	histone deacetylase
HLA	human leukocyte antigen
HOTAIR	HOX transcript antisense intergenic RNA
HOX	homeobox transcription factor
hTNF tg	human TNF transgenic
IκB	Inhibitor of κB
IKK	IκB kinase
IL	interleukin
IRAK	interleukin-1 receptor-associated kinase
JAK	janus kinase
JNK	Jun kinase
KAT	histone lysine acetyltransferase
kDa	kilo Dalton
KMT	histone lysine methyltransferase
lncRNA	long noncoding RNA
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemoattractant protein 1
Mϕ	macrophage
MHC	major histocompatibility complex
miRISC	miRNA-induced silencing complex
miRNA, miR	microRNA
MMP	matrix metalloproteinase
mRNA	messenger RNA
MTX	methotrexate
NF-κB	nuclear factor kappa B

NSAID	non-steroidal anti-inflammatory drug
nt	nucleotide
OA	osteoarthritis
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PRC	polycomb repressor complex
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA transcript
PTEN	phosphatase and tensin homologue
PTM	posttranslational modification
PTP4A3	protein tyrosine phosphatase type IV, member 3
qPCR	quantitative real-time PCR
RA	rheumatoid arthritis
RANKL	receptor activator of NF-κB ligand
RF	rheumatoid factor
RNA	ribonucleic acid
SD	standard deviation
SF	synovial fibroblast
siRNA	small interfering RNA
SLE	systemic lupus erythrematosus
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
SUZ12	Suppressor of Zeste 12 homologue (Drosophila)
TGF-β	transforming growth factor beta
Th	T helper cell
TLR	toll-like receptor
TNFα	tumour necrosis factor alpha
TNFAIP3	TNFα-induced protein 3 (A20)
TSS	transcription start site
UTR	untranslated region
VEGF	vascular endothelial growth factor
Wnt	wingless-type MMTV integration site
XIAP	X-linked inhibitor of apoptosis

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